



**CELESTINA GOMES
LOPES**

**CARATERIZAÇÃO DO PERFIL LIPÍDICO DE *Bacillus
licheniformis* I89 E DA SUA ALTERAÇÃO EM
RESPOSTA ÀS CONDIÇÕES DE CRESCIMENTO**

**CHARACTERIZATION OF THE LIPID PROFILE OF
Bacillus licheniformis I89 AND ITS CHANGES IN
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro

Apoio financeiro da Fundação Calouste Gulbenkian, através de uma Bolsas de investigação para Pós-graduação e Especialização destinados a Estudantes PALOP



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Dedico este trabalho à minha adorada mãe.

o júri

presidente

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professora associada com agregação da Universidade de Aveiro

agradecimentos

Escrever a tese não foi uma tarefa fácil, não é minha intenção aborrecer-vos com os motivos subjacentes e, sem o apoio das pessoas que passo a enumerar, provavelmente ainda me encontraria a trabalhar nela. Passo, assim, a expressar os meus sinceros agradecimentos.

À Professora Rosário Domingues e à Professora Sónia Mendo pela orientação deste trabalho, pelos conhecimentos transmitidos, pela disponibilidade e pela motivação. Mas acima de tudo, por me terem acolhido no vosso grupo de investigação e me terem oferecido a possibilidade de trabalhar neste projeto. Sou muito grata por tudo.

Aos restantes coautores dos trabalhos apresentados nesta tese pelos seus contributos.

Aos colegas do grupo de Espectrometria de Massa e do Laboratório de Biotecnologia Molecular do DBio pelo auxílio e pelo agradável ambiente de trabalho, e em particular à Joana Barbosa pela sua disponibilidade, acolhimento e conhecimento transmitidos. À Dra. Cristina Barros pelo apoio no laboratório. Um especial obrigado à Lara, à Emeline, à Stephanie, à Cesária, à Eveline Brito, à Fernandina e o Malam Sanha que foram o meu porto de abrigo durante estes anos.

Um especial obrigado a todos os amigos que de alguma forma me incentivaram a ser resiliente.

À minha mãe, os meus irmãos e os sobrinhos pelo amor e apoio incondicional.

À unidade de investigação de Química Orgânica, Produtos Naturais e Agroalimentares (QOPNA) (UID/QUI/00062/2019) e à Rede Nacional de Espectrometria de Massa (REDE/1504/REM/2005) da Universidade de Aveiro. Ao Centro de Estudos do Ambiente e do Mar (CESAM) (UID/AMB/50017/2019), da Universidade de Aveiro.

À Fundação Calouste Gulbenkian pelo financiamento através de uma Bolsa de investigação para Pós-graduação e Especialização destinados a Estudantes Africanos de Língua Portuguesa e de Timor-Leste sem o qual este trabalho não teria sido possível.

palavras-chave

Bacillus licheniformis I89, Ácidos gordos, Espectrometria massa, Bactéria de Gram positivo, Fosfolípidos e Glicolípidos.

resumo

Bacillus licheniformis I89 é uma bactéria de Gram positivo formadora de endósporos, que possui a capacidade de produzir vários compostos de interesse biotecnológico incluindo proteases, amilases, surfactantes e antibacterianos. Não existe informação sobre a composição lipídica desta estirpe. Alguns estudos publicados sobre outras espécies *B. licheniformis* reportam apenas a composição em ácidos gordos. Entre os ácidos gordos identificados, estão descritos ácidos gordos ramificados, também encontrados em outras bactérias de Gram positivo. Estes ácidos gordos ramificados estão descritos como sendo benéficos para a saúde humana e úteis na prevenção de doenças, para além das potenciais aplicações biotecnológicas. Assim, o presente trabalho teve como objetivo alargar o conhecimento sobre o lipidoma de *B. licheniformis* I89, nomeadamente a) identificar o perfil de ácidos gordos de estirpe *B. licheniformis* I89 e analisar a adaptação do perfil de ácidos gordos em função da temperatura de crescimento (37 e 50 °C) e nas diferentes fases do crescimento (lag, exponencial e estacionária), b) caracterizar o lipidoma de *B. licheniformis* I89 nas diferentes fases de crescimento (lag, exponencial e estacionária) a 37 °C, c) avaliar o efeito do antibiótico vancomicina na alteração do perfil de lípidos de *B. licheniformis* I89, nas fases lag e exponencial de crescimento a 37 °C. Para alcançar os objetivos propostos foram utilizadas as metodologias de cromatografia gasosa acoplada à espectrometria de massa (GC-MS) para identificação e quantificação de ácidos gordos e a cromatografia líquida de fase normal, usando uma coluna de interação hidrofílica, acoplada a espectrometria de massa (HILIC-ESI-MS) e MS/MS, para a identificação do perfil de lípidos polares. As mesmas metodologias foram também aplicadas para analisar o efeito de antibiótico vancomicina na alteração de perfil de lípidos da estirpe. O perfil de ácidos gordos (FAs) de *B. licheniformis* I89 obtido por GC-MS revelou a predominância de FAs ramificados das séries iso e anteiso (i-15:0, ai-15:0, i-16:0, i-17:0 e ai-17:0) e menor quantidade de ácidos gordos saturados (14:0, 16:0 e 18:0) em todas as condições de crescimento. O perfil de FAs variou com a temperatura e também com as fases de crescimento. Da fase lag para a fase estacionária, a 50 °C, houve uma diminuição dos ácidos gordos ai-17:0 e i-16:0, enquanto que o ácido gordo i-15:0 aumentou. Para a temperatura de 37 °C, observou-se um aumento dos ácidos gordos i-15:0 e i-16:0 e uma diminuição dos ácidos gordos ai-15:0 e ai-17:0. A análise do extrato lipídico por HILIC-ESI-MS e MS / MS permitiu identificar o lipidoma de *B. licheniformis* I89, o qual ainda não tinha sido descrito até a data.

resumo

No lipidoma de *B. licheniformis* I89 foram identificadas quatro classes de fosfolípidos: fosfatidiletanolamina, fosfatidilglicerol, lisil-fosfatidilglicerol e cardiolipina; duas classes de glicolípidos: monoglicosíldiacilglicerol e diglicosíldiacilglicerol; e duas classes de fosfogliceroglicolípidos: primer de ácido lipoteicóico monoalanilado e primer de ácido lipoteicóico. Todas as classes de lípidos foram identificadas nas três fases de crescimento analisadas, tendo-se observado variações na abundância em algumas espécies moleculares. Entre as fases exponencial e estacionária observou-se um aumento significativo nas espécies lipídicas 30:0 e uma diminuição significativa nas espécies lipídicas 32:0, quando comparadas com as da fase lag. Para além disso, estudou-se ainda a alteração na composição do perfil lipídico de *B. licheniformis* I89 na presença de vancomicina nas duas fases de crescimento (lag e exponencial) a 37 °C. Os resultados obtidos permitiram observar uma redução de algumas espécies moleculares de fosfatidilglicerol (PG), em resposta à presença da vancomicina. Uma vez que *B. licheniformis* I89 é sensível a este antibiótico, esta alteração pode ser justificada pelo facto da vancomicina atuar ao nível da inibição da síntese da parede celular podendo, por isso, afetar os lípidos que a constituem. Neste contexto, a abordagem lipidómica revelou-se uma ferramenta muito promissora no estudo da composição lipídica bacteriana, uma vez que esta permite analisar com precisão as alterações do perfil lipídico em resposta a diferentes condições de crescimento, nomeadamente, as que se observam na presença de antibióticos. Por outro lado, e dado a sua composição em ácidos gordos iso e anteiso, esta espécie de *Bacillus* pode vir a ser usada como fonte de ácidos gordos ramificados, uma vez que estes têm sido descritos como podendo ter atividade anti-tumoral. Muito embora essa atividade ainda tenha que ser investigada, os resultados obtidos fazem prever uma possível aplicação biotecnológica destes compostos com atividades terapêuticas.

keywords

Bacillus licheniformis I89, Lipidomic, Fatty acids, polar lipids, Mass spectrometry, Gram-positive bacteria, Phospholipids, Glycolipids.

abstract

Bacillus licheniformis I89 is a Gram-positive endospore-forming bacterium that has the ability to produce various compounds with biotechnological application including proteases, amylases, surfactants and antibacterial. There is no information on the lipid composition of this strain. Some published studies on *B. licheniformis* species report the composition in fatty acids. However, some branched fatty acids, already described in Gram positive bacteria, have also been detected and are predominant constituents of *B. licheniformis* I89. These branched fatty acids have been described as beneficial to human health and useful in disease prevention, and thus have potential biotechnological applications. In the present work we studied the lipidome of *B. licheniformis* I89. The main objectives of the study were: a) to identify the fatty acid (FA) profile of *B. licheniformis* strain I89 and to evaluate the adaptation of the fatty acid profile in response to the growth temperature (37 and 50°C) and in the different growth phases (lag, exponential and stationary), b) characterize the lipidome of *B. licheniformis* I89 in the different phases of growth (lag, exponential and stationary) at 37 °C and c) evaluate the effect of the antibiotic vancomycin on the alteration of the lipid composition profile of *B. licheniformis* I89 in the lag and exponential growth phases at 37 ° C. In order to reach the proposed objectives, gas chromatography coupled to mass spectrometry (GC-MS) was used to identify and quantify the FAs and normal phase liquid chromatography using a hydrophilic interaction column coupled to mass spectrometry (HILIC-ESI-MS) and MS/MS was used to identify the polar lipid profile. In addition, GC-MS and HILIC-ESI-MS were also used to analyze the effect of the antibiotic vancomycin on the lipid profile change. The FAs profile of *B. licheniformis* I89 obtained by GC-MS revealed the predominance of branched FAs of the iso and anteiso series (i-15:0, ai-15:0, i-16:0, i-17:0 e ai-17:0) and a low abundance of saturated FAs (14:0, 16:0 and 18:0) in all the growth conditions.

abstract

The FA profile showed variation with temperature and also with the growth phases. From lag phase to stationary phase at 50 ° C, there was a decrease of the FAs ai-17: 0 and i-16:0, while the FA i-15: 0 increased, whereas at 37 ° C, there was an increase of FA i-15:0 and i-16: 0 and a decrease of the FA ai-15:0 and ai-17:0. On the other hand, the lipidome of *B. licheniformis* I89 was identified for the first time by HILIC-ESI-MS and MS/MS. In the lipidome of *B. licheniformis* I89, four classes of phospholipids were identified: phosphatidylethanolamine, phosphatidylglycerol, lysylphosphatidylglycerol and cardiolipin; two classes of glycolipids: monoglycosyldiglycerol and diglycosyldiglycerol; and two classes of phosphoglyceroglycolipids: mono-alanylated lipoteichoic acid primer and lipoteichoic acid primer. All lipid classes were identified in the three growth phases analyzed, with a significant increase in the lipid species with 30:0 and a significant decrease in the lipid species with 32:0, between the exponential and stationary phases, when compared to the lag phase. In addition, a change in the composition of the lipid profile of *B. licheniformis* I89 was observed in the presence of vancomycin in the two growth phases (lag and exponential) at 37 ° C with the reduction of the levels of some PG molecular species. Vancomycin acts at the level of inhibition of the synthesis of the cell wall. *B. licheniformis* I89 is sensitive to this antibiotic and therefore, it seems to affect the membrane lipids. In this particular context the lipidomic approach employed is a very promising tool to study bacterial lipid composition. Since this allows to accurately analyze changes in lipid profile in response to different growth conditions, namely, those observed in the presence of antibiotics. Branched fatty acids have been described as having antitumor activity. Considering that *B. licheniformis* I89 is rich in branched fatty acids, this bacterium may be used as the source of this type of FA. However, it is still necessary to investigate a possible biotechnological application of these compounds as therapeutic agents.

Contents

List of figures.....	xxiii
List of tables.....	xxvi
Abbreviations.....	xxvii

CHAPTER I- INTRODUCTION

General Introduction	3
I.1 The genus <i>Bacillus</i>	4
I.2 <i>Bacillus licheniformis</i> , a species with interesting biotechnological applications.....	4
I.3 The composition and role of lipids in the Gram-positive bacterial cell wall	6
I.4 Methods used in lipid analysis in Gram-positive bacterium	9
I.4.1 Analytical approaches in the study of lipid in bacteria	9
I.4.1.1 Analysis of lipid classes of Gram-positive bacteria by TLC	13
I.4.1.2 Analysis of the fatty acid profile of Gram-positive bacteria by GC-FID and GC-MS.....	14
I.4.1.3- Analysis of the lipid profile of Gram-positive bacteria by mass spectrometry-based approaches	15
I.4.1.4 Lipidomic profile typical of Gram-positive bacteria using LC.....	18
I.5 The aim of the work	20
I.6 References	21

CHAPTER II – DECODING THE FATTY ACID PROFILE OF *Bacillus licheniformis* I89 AND ITS ADAPTATION TO DIFFERENT GROWTH CONDITIONS TO INVESTIGATE POSSIBLE BIOTECHNOLOGICAL APPLICATIONS.

II.1 Abstract.....	32
II.2 Introduction.....	32
II.3 Materials and Methods.....	33
II.3.1 Bacteria and growth conditions.....	33

II.3.2 Lipid extraction.....	34
II.3.3 Quantification of phospholipids by phosphorus assay.....	34
II.3.4 Fatty acid analysis by gas chromatography-mass spectrometry (GC-MS)	34
II.3.5 Statistical analysis.....	35
II.4 Results.....	35
II.4.1 FA Profile of <i>B. licheniformis</i> I89.....	35
II.4.2 FA Profile at Different Growth Temperatures	36
II.4.3 FA Profile at Different Growth Phases	37
II.4.4 Discussion.....	37
II.4.5 References.....	39

CHAPTER III- LIPIDOMIC SIGNATURE OF *Bacillus licheniformis* I89 DURING THE DIFFERENT GROWTH PHASES UNRAVELLED BY HIGH-RESOLUTION LIPID CHROMATOGRAPHY-MASS SPECTROMETRY

III.1 Abstract.....	43
III.2 Introduction.....	43
III.3 Materials and Methods.....	44
III.3.1 Bacteria and growth conditions.....	44
III.3.2 Lipid extraction.....	44
III.3.3 Quantification of phospholipids by phosphorus assay.....	44
III.3.4 Hydrophilic interaction liquid chromatography - electrospray ionization - mass spectrometry (HILIC-ESI-MS)	45
III.3.5 Data and Statistical analysis.....	45
III.4 Results.....	47
III.4.1 Phospholipid profile.....	47
III.4.2 Glycolipid profile.....	47
III.4.3 Phosphoglyceroglycolipid profile.....	49
III.4.4 Lipid profile is growth phase-dependent.....	49
III.5 Discussion.....	49
III.6 Conclusions.....	51

III.7 References.....	52
-----------------------	----

CHAPTER IV- CHANGES IN *Bacillus licheniformis* I89 MEMBRANE LIPID COMPOSITION AFTER EXPOSURE TO VANCOMYCIN

IV .1 Introduction.....	57
IV .2 Materials and Methods.....	58
IV.2.1 Determination of minimum inhibitory concentration for vancomycin (MIC).....	58
IV.2.2 Growth of <i>B. licheniformis</i> I89 in the presence of vancomycin.....	58
IV.2.3 Lipid extraction.....	59
IV.2.4 Quantification of phospholipids by phosphorus assay.....	60
IV.2.5 Fatty acid analysis by gas chromatography-mass spectrometry (GC-MS).....	60
IV.2.6 Hydrophilic interaction liquid chromatography - electrospray ionization - mass spectrometry (HILIC-ESI-MS)	61
IV.2.7 Data and Statistical analysis.....	62
IV.3 Results.....	63
IV.3.1 MIC determination and establishment of vancomycin assay conditions...	63
IV.3.2 Adaptation of FA profile from <i>B. licheniformis</i> I89 in the presence of vancomycin	63
IV.3.3 Adaptation of polar lipid profile from <i>B. licheniformis</i> I89 in the presence of vancomycin.....	65
IV.4 Discussion.....	70
IV.5 Concluding Remarks.....	72
IV.6 References.....	73

CHAPTER V- GENERAL CONCLUDING REMARKS78

List of figures

CHAPTER I

- Fig. I.1** Structure of the lantibiotic lichenicidin which is composed of two peptides Bli α and Bli β5
- Fig. I.2** - General structure of the Gram-positive cell wall.....6

CHAPTER II

- Fig. II.1** Main classes of fatty acids identified in *B. licheniformis* I89: a) Saturated fatty acids represented here by 15:0; Branched fatty acids represented here by b) i-15:0, c) ai-15:0.....33
- Fig. II.2** Principal coordinates analysis of the fatty acid composition of *Bacillus licheniformis* I89 in growth phase and growth temperature at 37 °C and at 50 °C.....36
- Fig. II.3** Comparison of the variation of fatty acid profiles of *Bacillus licheniformis* I89 in each growth phase, depending on the growth temperature: lag at 37 °C and at 50 °C (a), exponential (Exp) at 37 °C and at 50 °C (b) and stationary (Sta) 37 °C and at 50 °C (c). Values are means \pm standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$36
- Fig. II.4** Comparison of the fatty acid profiles of *Bacillus licheniformis* I89 between the lag, exponential (Exp) and stationary (Sta) growth phases at 37 °C (a) and at 50 °C (b). Values are means \pm standard deviation, * $p < 0.05$, ** $p < 0.01$37

CHAPTER III

- Fig. III.1.** HILIC-ESI-MS chromatograms of total lipid extracts of *B. licheniformis* I89 in (a) negative ion mode and (b) positive ion mode and the retention time (RT) of each polar lipid class. PG: Phosphatidylglycerol, RT 2.3 min; CL: Cardiolipin, RT 2.3 min; LTAP: Lipoteichoic acid primer, RT 2.5 min; DGDG: Diglycosyldiacylglycerol, RT 3.0 min; MGDG: Monoglycosyldiacylglycerol, RT 3.0 min; LTAP-Ala: Mono-alanylated

lipoteichoic acid primer, RT 4.0 min; PE: Phosphatidylethanolamine, RT 5.1 min; lys-PG: lysyl-phosphatidylglycerol, RT 15.7 min.....	44
Fig. III.2 Chemical structures of the polar lipids identified in <i>B. licheniformis</i> I89.....	45
Fig. III.3 LC-MS spectra of the phospholipid classes identified in <i>B. licheniformis</i> I89 lipidome in the negative ion mode: PG (a), lys-PG (c), CL (e) and in positive ion mode for PE (g). LC-MS/MS spectra and fragmentation pattern of one of the possible isomers of the $[M - H]^-$ ions of PG (17:0/15:0) at m/z 721.5 (b), lys-PG (17:0/15:0) at m/z 849.6 (d), CL (15:0/15:0/15:0/17:0) at m/z 1323.9 (f) and of the $[M + H]^+$ ion of PE (17:0/15:0) at m/z 692.3 (h).....	48
Fig. III.4 LC-MS spectra of glycolipids identified in <i>B. licheniformis</i> I89 lipidome: MGDG (a) and DGDG (c). LC-MS/MS spectra acquired in positive ion mode and fragmentation pattern of one of the possible isomers of MGDG (17:0/15:0) at m/z 748.6 (b) and DGDG (17:0/15:0) at m/z 910.6 (d).....	50
Fig. III.5 LC-MS spectra of phosphoglyceroglycolipids identified in <i>B. licheniformis</i> I89: LTAP (a) and LTAP-Ala (c). LC-MS/MS spectra acquired in negative ion mode and fragmentation pattern of one of the possible isomers of the $[M - H]^-$ ions LTAP (15:0/15:0) at m/z 1017.6 (b) and LTAP-Ala (17:0/15:0) at m/z 1116.6 (d).....	51
Fig. III.6 Comparison of the polar lipid species of <i>B. licheniformis</i> I89 between the lag, exponential (Exp) and stationary (Sta) growth phases at 37 °C: (a) PG, (b) lys-PG, (c) CL (d) PE, (e) DGDG, (f) MGDGD, (g) LTAP and (h) LTAP-Ala. Values are means \pm standard deviation, * $p < 0.05$, *** $p < 0.01$ and *** $p < 0.001$	52

Chapter IV

Fig. IV.1 Variation of FA composition of <i>B. licheniformis</i> I89 membrane in the lag (a) and exponential (b) phases, after exposure to vancomycin at concentrations of 0.008 and 0.125 $\mu\text{g/mL}$. Values are means \pm standard deviation, * $p < 0.05$	64
Fig. IV. 2 Variation of <i>B. licheniformis</i> I89 membrane polar lipid composition of the classes PG, lys-PG, PE and DGDG in the lag phase, after exposure to vancomycin in concentrations of 0.008 and 0.125 $\mu\text{g/mL}$. Variation was observed for the polar lipid classes PG (a), lys-PG (b) PE (c), and DGDG (d). Values are means \pm standard deviation, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$	67

Figure IV.3 Variation of the polar lipid composition of *B. licheniformis* I89 membrane in the exponential phase, after exposure to vancomycin in concentrations of 0.008 and 0.125 µg/mL. Variation was observed for the polar lipid classes lys-PG (a) and PE (b), Values are means ± standard deviation, ** p < 0.0169

APPENDIX SUPPLEMENTARY MATERIAL OF SECTION III

Supplementary fig. III.1 LC-MS spectra of the phospholipid classes (PLs) acquired in positive ion mode for PG (a), lys-PG (c), and in negative ion mode for PE(e) and CL(g), identified in *Bacillus licheniformis* I89 lipidome. LC-MS/MS spectra and possible fragmentation pattern of one of the possible isomers of the $[M + NH_4]^+$ ions of PG (17:0/15:0) at m/z 740.5(b), $[M + H]^+$ ion of Lys-PG (17:0/15:0) at m/z 851.6 (d), $[PE - H]^-$ ion of PE (17:1/15:0) at m/z 690.3(f) and $[CL - 2H]^{2-}$ ion of CL (15:0/17:0) m/z 661.5(h).....80

Supplementary figure III.2 LC-MS spectra of the glycolipid classes (GLs) acquired in negative ion mode for MGDG (a), DGDG (c) identified in *Bacillus licheniformis* I89 lipidome. LC-MS/MS spectra and possible fragmentation pattern of one of the possible isomers of the $[M - CH_3COO]^-$ ions of MGDG (17:0/16:0) at m/z 775.6 (b) and DGDG (15:0/16:0) m/z 937.6(d).....81

List of tables

Chapter I

Table I.1 Summary at the results work using MS- approaches for the identification in lipids composition of Gram-positive bacteria reported in the literature.....10

Table I.2 Typical specific fragmentation pathway (product ions and neutral loss (NL)) of polar lipid from Gram-positive bacteria identified in the MS/MS spectrum.....16

Chapter II

Table II.1 Variation of the fatty acid profiles of *Bacillus licheniformis* I89 in each growth phase, depending on the temperature: lag at 37 °C and at 50 °C, exponential (Exp) at 37 °C and at 50 °C and stationary (Sta) 37 °C and at 50 °C and the lag, exponential and stationary growth phases at 37 °C and at 50 °C.....35

Table II.2 Similarity percentage analysis (SIMPER) identifying which FA contributing to the differences recorded at the growth temperatures of 37 °C and at 50 °C in *Bacillus licheniformis* I89.....36

Chapter III

Table III.1 Molecular species of phospholipids from *B. licheniformis* I89 identified by LC-MS in positive and negative ion modes. The iso and anteiso C15 and C17 are the most abundant FAs of *B. licheniformis* I89. It is not possible to distinguish the linear C15 and C17 from their branched isomers by LC-MS and MS/MS analysis.....46

Table III.2 Molecular species of glycolipids from *B. licheniformis* I89 identified by LC-MS in positive and negative ion modes. The iso and anteiso C15 and C17 are the most abundant FAs of *B. licheniformis* I89. It is not possible to distinguish the linear C15 and C17 from their branched isomers by LC-MS and MS/MS analysis.....49

Table III.3 Molecular species of phosphoglyceroglycolipids from *B. licheniformis* I89 identified by LC-MS in negative ion modes. The iso and anteiso C15 and C17 are the most

abundant FAs of *B. licheniformis* I89. It is not possible to distinguish the linear C15 and C17 from their branched isomers by LC-MS and MS/MS analysis.....50

List of Abbreviations

<i>B. licheniformis</i>	<i>Bacillus licheniformis</i>
CL	Cardiolipin
ESI-MS	Electrospray ionization mass spectrometry
GC- FID	Gas chromatography - flame ionization detector
GC- MS	Gas chromatography - mass spectrometry
GL	Glycolipids
LC	Lipid chromatography
Lys-PG	Lysyl-phosphatidylglycerol
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGL	Phosphoglyceroglycolipids
TA	Teichoic acid
FID	Flame ionization detector
MS	Mass spectrometry
HPLC	High performance liquid chromatography,
HILIC	Hydrophilic interaction chromatography
UHPSFC	Ultrahigh performance supercritical fluid chromatography
LTAP	Lipoteichoic acid primer
LTAP-Ala	Mono-alanylated lipoteichoic acid primer
HILIC-ESI-MS	Hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometry
MS/MS	Tandem mass spectrometry
MGDG	Monoglycosyldiacylglycerols
DGDG	Diglycosyldiacylglycerols
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight

CHAPTER I. INTRODUCTION

GENERAL INTRODUCTION

I.1 THE GENUS *BACILLUS*

I.2 *Bacillus licheniformis*, A SPECIES WITH INTERESTING BIOTECHNOLOGICAL APPLICATIONS

I.4 THE COMPOSITION AND ROLE OF LIPIDS IN THE GRAM-POSITIVE BACTERIAL CELL WALL

I.5 METHODS USED IN LIPID ANALYSIS IN GRAM-POSITIVE BACTERIUM

I.5.1 ANALYTICAL APPROACHES IN THE STUDY OF LIPID IN BACTERIA

I.5.1.1 ANALYSIS OF LIPID CLASSES OF GRAM-POSITIVE BACTERIA BY TLC

I.5.1.2 ANALYSIS OF THE FATTY ACID PROFILE OF GRAM-POSITIVE BACTERIA BY GC-FID AND GC-MS

I.5.1.3 ANALYSIS OF THE LIPID PROFILE OF GRAM-POSITIVE BACTERIA BY MASS SPECTROMETRY-BASED APPROACHES

I.5.1.4 LIPIDOMIC PROFILE TYPICAL OF GRAM-POSITIVE BACTERIA USING LC-MS

I.6 THE AIM OF THE WORK

General Introduction

Bacteria comprises a large group of microorganisms with interest in the different fields, not only due to their effect in health, disease prevention, and treatment but also as pathogens. Bacteria can produce many compounds of interest with several biotechnological applications [1]. Bacteria have the ability to adapt to different growth conditions including temperature and pH which make them attractive to be used in industrial processes, since growth conditions can be adjusted and manipulated to improve the production of the biotechnological compound(s) of interest [2]. The bacterial lipid composition varies between species, environmental conditions, and growth phase [3–5]. Lipids seem to play an important role in the adaptation process and in the metabolism of bacteria. Nevertheless, the adaptation of lipid metabolism and the variation of lipid composition in bacteria in response to different growth conditions is far from being completely understood. Lipids are the main components of cell membranes, and different bacteria display different lipid composition [6]. Fatty acid profile is quite specific among different bacteria [7]. Nonetheless, it is recognized that under different growth temperatures, a shift in the fatty acid profile is observed [4]. Nonetheless, fatty acids are usually esterified to other lipids, such as phospholipids and glycolipids that are in fact the main constituents of the cell membranes and the cell wall of bacteria. But the knowledge of the composition and importance of polar lipids is far from being completely understood mainly because the bacterial lipidome is quite complex, and the lipidome of most bacteria remains unknown. Only in the last decade, with the use of modern mass spectrometry-based approaches, new studies have addressed the total lipidome of bacteria at the molecular level. This will contribute with new knowledge and understanding as regards the role of lipids in bacteria, with benefits for the discovery of new antimicrobials, or new biotechnological application of bacteria, their constituents and their products. The present work addresses the lipidome of *B. licheniformis* I89, a non-pathogenic Gram-positive bacterium producer of the lantibiotic (lanthipeptides with antibacterial activity) lichenicidin and other compounds with biotechnological interest.

I.1 The genus *Bacillus*

Bacillus is a genus of Gram-positive bacteria. It comprises rod-shaped endospore-forming bacterium with an aerobic or facultative anaerobic metabolism [8]. Members of the genus *Bacillus* have been isolated from a wide variety of environments such as plant and soil [9–11]. Some *Bacillus* species are pathogenic, yet many others are of industrial, and commercial interest [12]. Among the pathogenic species are *B. anthracis*, the causative agent of anthrax in humans and other animals, and *B. cereus*, which is a common cause of food poisoning [13]. *B. thuringiensis* and some strains of *B. cereus* are pathogenic to certain insects. Other *Bacillus* species have special characteristics that make them good candidates as biological control agents (fungicides, bactericides, and fertilizers) [14,15]. With regard to species with industrial applications [16], *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* have a long history of safe commercial application in the food, detergents and pharmaceuticals industries [17]. Due to their applications, *Bacillus* species are of great economic interest and are of value to the biotechnology industry.

I.2. *Bacillus licheniformis*, a species with interesting biotechnological applications

Bacillus licheniformis is a non-pathogenic, Gram-positive endospore-forming bacterium frequently found in soil. It is a facultative anaerobe bacterium that belongs to the *B. subtilis* group II [18,19]. Members of this genus have been described as having several biotechnological applications as produces of valuable compounds such as proteases, amylases, surfactants, lantibiotics, biofuels, and other secondary metabolites [20]. It is usually considered safe, and some strains of this species are considered as probiotic and are used in the food and feed industry [12]. Also, this species produces several compounds of interest. Bacitracin is a peptide antibiotic produced by *B. licheniformis* with activity against Gram-positive bacteria, a few Gram-negative such as meningococci and gonococci and also against gas gangrene infection in guinea pigs and haemolytic streptococcal infection [21]. Lichenysin A, a cytotoxic substance, is a small cyclic lipopeptide structurally very similar to surfactants, also produced by *B. licheniformis*, exhibits antibacterial activity. It is not commercialized as antibacterial due of its toxicity [22]. Some *B. licheniformis* isolates can

mitigate the effects of fungal pathogens on maize, grasses and vegetable crops [19]. Other applications can be found for *B. licheniformis*. Its great fermentative capacity combined with low toxicity has made *B. licheniformis* 2336 a popular choice as probiotics [23]. In another recent study, antimicrobial compounds derived from *B. licheniformis* BFP011 isolated from papaya (Thailand) were shown to inhibit the growth of several important phytopathogens, as well as human pathogenic and food spoilage bacteria, namely, *Colletotrichum capsici*, *Escherichia coli* O157: H7 and *Salmonella typhi* ATCC 5784 [24]. The proteases from *B. licheniformis* are used in the detergent industry as well as for dehairing and bating of leather [25] and amylases. produced by this species are employed for the hydrolysis of starch, desizing of textiles and sizing of paper [19]. The antagonistic compounds, bacillocin from *B. licheniformis* is able to inhibit the growth of Gram-positive isolates such as *B. anthracis*, *L. innocua*, *S. epidermidis* [26]. Apart from peptides, polyketides are another important family of secondary metabolites produced from *B. licheniformis* that have also antimicrobial, immunosuppressive, antitumor or other physiologically relevant bioactivities. Although polyketides are widespread secondary metabolites from bacteria, only a few have been isolated and characterized from *Bacillus* [27]. In addition, the *B. licheniformis* I89 strain that will be studied in the present thesis was also reported as a producer of other compounds with biological interest. This strain, isolated from a hot spring environment in São Miguel, Azores, Portugal [28], is producer of lichenicidin, a two-component lantibiotic consisting of two peptides, Bli α and Bli β (Fig 1) [29] with activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *L. monocytogenes* [30,31].

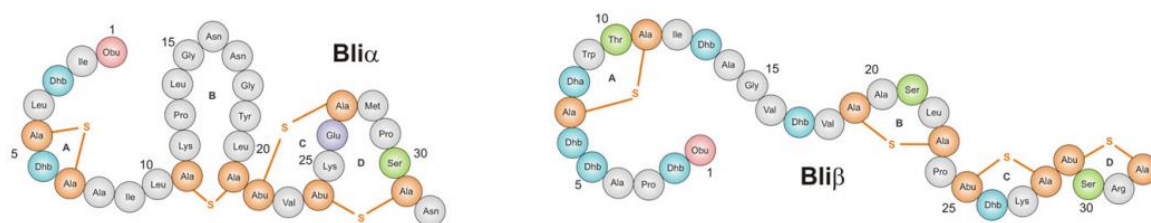


Fig. I.1 Structure of the lantibiotic lichenicidin which is composed of two peptides Bli α and Bli β [32].

Considering the interest and wide applications of *B. licheniformis* in the industry, in the manufacture of various biotechnological relevant products, it will be interesting to deepen the knowledge of the lipid composition of this bacterium. The information retrieved can be useful in searching for new applications of these molecules in other less explored areas of biotechnology.

I.3 The composition and role of lipids in the Gram-positive bacterial cell wall

Gram-positive bacteria cell wall is a complex structure (Figure 1). The cell wall comprises a thick peptidoglycan layer of about 40-80 nm that stabilizes the cell membrane [33]. In addition, the cell wall of Gram-positive bacteria contains a unique component, the teichoic acid (TA), that can be covalently attached to the *N*-acetylmuramic acid unit of peptidoglycan (wall teichoic acids, WTAs) or anchored to a glycolipid to the cytoplasmic membrane (lipoteichoic acids, LTAs) [34,35]. The TA or related glycopolymer play crucial roles in bacterial survival under disadvantageous conditions and in other basic cellular processes such as protection against harmful substances and environmental stresses, control of enzyme activities and cation concentrations in the cell envelope, and binding to receptors and surfaces [34]. Gram-positive bacteria lack the outer membrane as well as phosphate-rich lipid A and lipopolysaccharide found in Gram-negative bacteria.

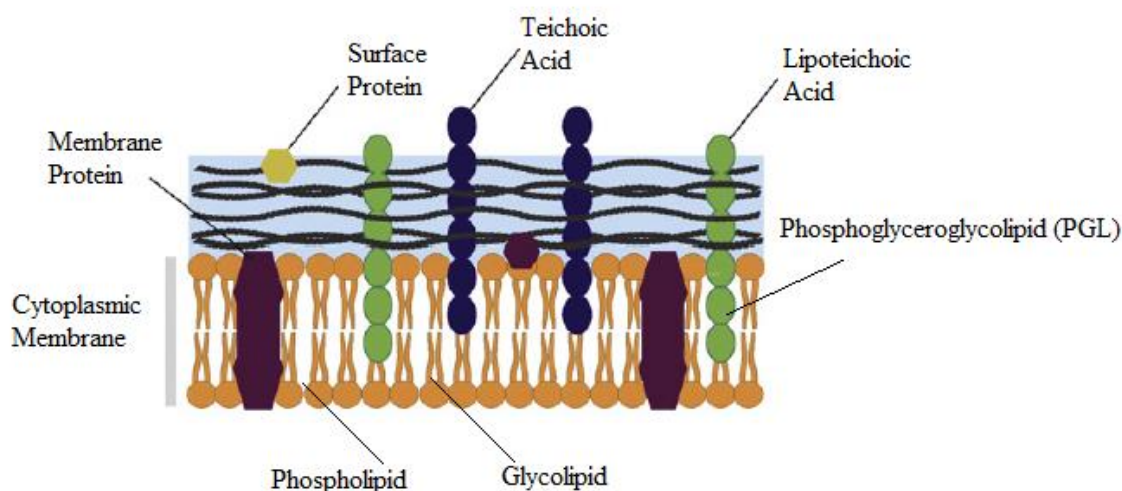


Fig. I.2 General structure of the Gram-positive cell wall adapted [36].

The bacterial cytoplasmic membrane forms a crucial barrier to the cell. It is composed of a glycerolipid bilayer with associated protein molecules and is very important for cell physiology, homeostasis, and survival. The biophysical properties of the membrane are, to a large extent, determined by the fatty acyl residues of the membrane and also by their composition in phospholipids and glycolipids [3,37].

Lipids are major compounds of bacteria cell membranes and share a large variety of biological functions. In bacteria, most lipids are located in the cell wall, mainly in the cytoplasmic membrane [38]. Phospholipids are the major constituent of the cell membrane and play a fundamental role in the maintenance of the membrane integrity, fluidity and charge that, in turn, modulate interactions with membrane-associated proteins [3,39]. The presence of anionic phospholipids renders bacterial cell surface negatively charged. This feature makes bacterial membrane the easy target of host immune molecules such as cationic antibiotic peptides [40–42]. Some of these phospholipids are also signalling molecules and participate in signal transduction [3]. The bacterial cytoplasmic membrane is a heterogeneous highly dynamic structure, comprising diverse domains differing in their phospholipid and protein composition, giving rise to defined membrane microenvironments and play an important role in compartmentation of specific proteins in the membrane.[43]. Some lipids are also carriers of important nutrients such as fatty acids, important for cell metabolism.

Gram-positive bacteria contain branched fatty acids with the major distribution of anteiso C15:0 and C17:0 chains. The most prevalent FAs in bacterial lipids have chain lengths between C12 and C20 [44]. The FAs profile can be straight-chained, branched-chained, unsaturated and cyclopropane. The branched-chain fatty acids are typical of Gram-positive bacteria and can be used as a marker for the differentiation of Gram-negative and Gram-positive bacteria [7].

Lipids in the bacteria cell membrane are quite diverse and bacteria specific and have some phospholipid and glycolipids that are not found in mammals. In bacteria, there are three major classes of phospholipids, namely phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) [45,47]. Other phospholipids specific of Gram-positive bacteria were described. According to previous reports, Gram-positive species express at least one type of aminoacylated phosphatidylglycerol [37,45]. Unlike

Gram-negative bacteria, Gram-positive bacteria typically have less PE but have an abundance of lysyl-PG or other aminoacylated PGs which are absent in most Gram-negative bacteria [46,47]. The most common aminoacylated phosphatidylglycerol lipids found in bacteria are lysyl-phosphatidylglycerol (lysyl-PG) and also amino acyl esters including alanyl-, ornithyl-, or arg-PG, that have been found in several Firmicutes such as *B. subtilis* (lysyl-PG) [48,49], *S. aureus* (lysyl-PG) [50], and *L. monocytogenes* (lysyl-PG) [51,52], *B. subtilis* (alanyl-PG), [48,53], *C. perfringens* (alanyl-PG), *B. cereus* and *Mycobacterium* (ornithyl-PG), *E. faecium* and *E. faecalis* (arg-PG) [54]. Other serine and glycine-containing lipids are also known to exist in bacteria [55].

The presence of these amino acyl esters in the membrane reduces the susceptibility to antimicrobial peptides produced during host infection and their absence in the membrane of bacteria leads to hypersensitivity to antibiotics [56]. Besides, the phospholipid lysyl-PG is practically absent in Gram-negative bacteria. Phosphatidylinositol (PI) a phospholipid widely present in mammal cells is scarce in bacteria but it is found in a few Gram-positive species including *Listeria monocytogenes* [51]. Phosphatidic acid (PA) and phosphatidylserine (PS), are widespread in bacteria but they are present in small amounts [57]. Other types of polar lipids bearing phosphates groups found in Gram-positive bacteria are the lipo- and wall-teichoic acids found only in Gram-positive bacteria. Besides phospholipids, other lipids were reported in bacteria such as glycolipids (GL) and phosphoglyceroglycolipids (PGL), of which the last is only present in Gram-positive bacteria. PGL play crucial roles in cell shape determination, regulation of cell division, and other fundamental aspects of Gram-positive bacterial physiology and are important in pathogenesis and play key roles in antibiotic resistance [58]. Also, mannosides derivatives of phosphatidylinositol were found in Actinomycetes and in few other bacteria [6].

Different bacteria can have a different phospholipids composition, and their relative abundance in cell membranes can be bacteria specific. In Gram-positive bacteria belonging to the phylum Firmicutes, such as *B. subtilis*, *S. aureus*, and *L. monocytogenes*, PG is the main membrane component of the total phospholipid pool being PE the second most abundant class, followed by CL and Lys-PG [59].

In bacteria, the composition in phospholipids, either the proportion of the PL classes and their composition in fatty acyl chains can change rapidly in response to environmental conditions, such as extreme temperatures, osmotic stress or low pH [3]. The content in

branched fatty acids in membranes can vary substantially depending on the growth conditions. Membrane lipid composition affects membrane viscosity, which modulates membrane permeability and can influence both solute transport and protein interactions. Membrane lipid homeostasis is thus a crucial process and can compromise bacteria viability [60]. Membrane defines the boundary between a single cell and its environment and is, therefore, the main target for antibacterial agents.

The knowledge on the composition of lipid components of both the cell wall and the bacterial membrane is thus important and have been addressed for many years, using different analytical approaches but, only recently, with the development of new methodologies, such as omics base mass spectrometry approaches, this area is reaching new developments, which will be addressed in the following chapters.

I.4 Methods used in lipid analysis in Gram-positive bacterium

The knowledge on the composition of lipid components of the cell wall and the bacterial membrane has been addressed using low technology methodologies as thin layer chromatography (TLC), and gas chromatography (GC) coupled with flame ionization detector (FID) or mass spectrometry (MS) or high-tech approaches, namely mass spectrometry-based approaches, using liquid chromatography coupled with mass spectrometry (LC-MS). Both have been applied to the study of lipid composition of Gram-positive bacteria and including *Bacillus* as will be described in this chapter, that will highlight the state of the art of the results obtained in the identification of the lipidome in Gram-positive bacteria.

I.4.1 Analytical approaches in the study of lipid in bacteria

Lipid analysis is a multi-step process that starts with the extraction of lipids, followed by the separation of lipids and analysis. Most of the methods reported in bacterial studies are based on the extraction of lipids using conventional methodologies such as Bligh and Dyer and Folch methods [49,61,70,71,62–69] (Table 1). These are, still nowadays, the most

popular methods used in the studies of lipids in bacteria. The total lipid extract obtained can be fractionated followed by analysis of fractions, or alternatively, the total lipid extract can be further analyzed. Fractionation can be done by thin layer chromatography (TLC), by solid phase extraction (SPE) or by liquid chromatography (LC). Total extracts or fractions can be analyzed by TLC, GC-FID, gas chromatography coupled with mass spectrometry (GC-MS) or LC-MS. The first approach, TLC, gives information about lipid class, the other following two approaches give information about the composition in fatty acids, while LC-MS is used to identify the total lipidome profile, with information at the molecular level and at the class level. Screening of bacterial lipids have been carried using these different MS methods (Table 1) and the information about these approaches and results will be detailed in the following subsections.

Table I.1 Summary at the results work using MS- approaches for the identification in lipids composition of Gram-positive bacteria reported in the literature.

Species of bacteria	Lipid Classes	Methods of Extraction	Methods at analysis	Ref.
<i>Bacillus</i>				
<i>B. subtilis</i>	PE, PG, Lys-PG, CL, GL, and GPL	Folch / Bligh and Dyer	2D TLC	[61]
<i>B. subtilis</i>	6PE, 9PG, 2Lys-PG, CL, and 3DGDG,	Folch / Bligh and Dyer	MALDI-TOF-MS and LIFT-TOF-TOF	[49]
<i>B. subtilis</i>	7PE, 4PG, 1Ly-PG, CL, and PA	Bligh and Dyer	GC-MS, LC-MS/MS, MALDI-TOF/TOF	[59]
<i>B. subtilis</i>	4PE, 8PG, PS and 6PA	Hexane-propan-2-ol mixture	TLC, LC-MS and GC-MS	[86]
<i>B. subtilis</i>	PG, CL, alanyl-PG, Lyso-CL,	Bligh and Dyer	QTRAP	[88]

	DGDG, LTAP, LTAP-Ala, and LTAP-(Ala) ₂			
<i>B. cereus</i>	PG, PE and CL	Bligh and Dyer	TLC and GC-MS	[62]
<i>B. stearotheophilus</i>	PG, PE, CL, and PGL	Bligh and Dyer	TLC and GC-MS	[63]
Enterococcus				
<i>E. faecalis</i>	17PG, 8Lysyl-PG, 23CL, 5DGDG, 3DAG, 4TAG and 3PGL	Bligh and Dyer	TLC, LC-MS/MS	[64]
Staphylococcus				
<i>S. aureus</i>	PG, PE, Lysyl-PG, and CL	Bligh and Dyer	TLC	[65]
<i>S. aureus</i>	PG, Lysyl-PG, CL, DGDG, MDGD, and DAG	Bligh and Dyer	RP-LC-Q-TOF-MS (Q-TOF-MS)	[66]
<i>S. warneri</i>	PG, CL, PC, PA, and Lysyl-PG	Bligh and Dyer	TLC/ESI-MS, HILIC-LC-MS, MS/MS	[67]
Mycoplasma				
<i>M. hyorhinis</i>	PG, CL, and GL	Bligh and Dyer	TLC	[74]
Clostridia				
<i>C. perfringens</i>	PG, PE, Lysyl-PG, and CL	Bligh and Dyer	2D TLC	[68]
<i>C. novyi</i>	PG, PGp, PE, PEp, Lysyl-PG, Lysyl-PG	Bligh and Dyer	2D TLC, LC-MS	[69]

	Alanyl-PG, PS, and PT			
<i>C. botulinum</i>	PG, PGp, PE, PEp, CL, CLp, Ala-PG and Lysyl-PG	Bligh and Dyer	2D TLC, LC-MS	[88]
<i>Listeria</i>				
<i>L. monocytogenes</i>	PG, CL, Lysyl-PG, Lysyl-CL and PI		TLC	[51]
<i>L. monocytogenes</i>	8PG, 6CL, 6Lysyl-CL and 6DGDG	Chloroform, methanol, and saline	LIT MS ⁿ	[52]
<i>Lactobacillus</i>				
<i>L. plantarum</i>	DGD and DGDG	Bligh and Dyer	ESI-MS/MS, 1D-, 2D-NMR, TLC, GC-MS	[70]
<i>Streptococcus</i>				
<i>S. pneumoniae</i>	23MGDG and 2DGDG	CHCl ₃ , CH ₃ OH, 0.3% NaCl	LIT/ MS ⁿ	[89]
<i>S. pneumoniae</i>	PG, CL, DGDG, and MDGD		TLC	[91]
<i>S. thermophilus</i>	DG and TG	Folch	TLC, GC/FID and GC/MS.	[75]

I.4.1.1 Analysis of lipid classes of Gram-positive bacteria by TLC

TLC is a technique used to analyze, identify or separate the components of a mixture. It allows rapid and relatively inexpensive separation of the phospholipid classes and can be used prior to mass spectrometry analysis [71]. But this method has several disadvantages, such as low resolution and sensitivity, increased oxidation of lipid compounds, and given limited information in lipidome analysis. TLC is usually used to separate the major classes of lipids such as the case of separation of phospholipid classes [72]. Separation and fractionation of lipid classes are based on the different polarity conferred by the different composition of the polar head groups. The identification of each class is based on the comparison with lipid standards applied to the same TLC plate. After the separation, each phospholipid class can be quantified, by determination of phosphorous in each spot. It requires that each spot corresponding to different classes be scrapped from the plate and used for the quantification of the phosphorus in each class [63]. Analysis of lipids by TLC allowed the identification and separation of phospholipid classes in some Gram-positive bacteria. In TLC analysis, lipid classes were identified and separated in PG, PE, lysyl-PG, CL and DGDG in *B. subtilis* [73], PE, PG, CL and PGL (phosphoglyceroglycolipid) in *B. stearothermophilus* [63], CL, PA, PG, Ceramide, PE, PC, PS in *S. warneri* [67], PG, CL, PE and lysyl-PG in *S. aureus* [65], PE, PG, CL, and lysyl-PG, Ala-PG and lyso-PG in *C. perfringens* [68], PE, PG, lysyl-PG, alanyl-PG, PS e phosphatidyl threonine (PT) and their plasmalogens in *C. novyi* ; MDGD, DGDG in *L. plantarum* ; PG, CL, DGDG and MGDG in *S. pneumoniae* [63]; in *L. monocytogenes* PG, CL, lysyl-PG, lysyl-CL [50], *M. hyorhinae* sphingomyelin (SPM), PG, PC, CL and neutral lipids (NL)[74] . However detailed structural information cannot be obtained. The lipids in each spot can be extracted using organic solvents and the respective extract can be analyzed by GC-FID or GC-MS or by MS to gain more structural information.

I.4.1.2 Analysis of the fatty acid profile of Gram-positive bacteria by GC-FID and GC-MS

The fatty acids are routinely analyzed in the form of respective esters by GC-FID, or GC-MS [7,75]. GC allows an excellent separation of fatty acids prior to analysis, but requires extensive sample preparation, for the chemical derivatization of the fatty acids. Typically, they are esterified into the methyl ester in order to provide enough volatility for the separation by the GC. Most of the published works describe the identification of the fatty acid profile from the total lipid extract, thus allowing to identify the components in the fatty profile of bacteria [62,63]. In fact, these works showed that different Gram-positive bacteria have a characteristic composition in FA that could be used for taxonomic discrimination purposes. GC-MS has been applied for the analysis of the FA profile in the differentiation of bacterial classes [7,76–78]. Analysis of FA profile has been widely employed and it is not the goal of our work to revise all data from Gram-positive bacteria, and thus we will focus only on the main findings related with FA composition of *Bacillus*.

GC-MS approaches have been widely used to study the FA profile of several *Bacillus* spp. Overall, the FAs identified included saturated, unsaturated and branched FA, with a chain length between C14 to C19. FA with C12 and C13 have been scarcely reported. For example the FA profile of *B. subtilis*, *B. stearothermophilus*, *B. simplex*, *B. vanillea* sp *Bacillus pumilus*, and *B. cereus* comprised mainly the FAs i-15, ai-15, i-17, ai-17, i-16, 16:0, 18:0 [4,62,63,79–81]. Some other minor FA is sometimes reported, in addition to the previous ones, as is the case of cyclo-16:0 in *Bacillus pumilus* [81]. Other Gram-positive bacterium such as *S. aureus* has, the following FA as the most abundant, the FAs i-14:0, i-15:, ai-15:0, i-16:0,16:0, i-17:0, ai-17:0, 18:0, i-19:0 ai-19 [82] and other less common FA such cys-18:1, 18:2 1-OH-12:0, tr-18:1, 2-OH-14:0 [83]. In the case of *L. monocytogenes* FAs i-15:0 ai-15:0 i-17:0 ai-17:0 were reported [84]. However, the percentage of each FA in the total FA profile is dependent on the species and on the growth conditions [36]. Most of the published works describe the FA profile and the composition of the total lipid extracts from bacteria. Other approaches have also been used, such as the TLC combined with GC-MS. In this approach, TLC allows to fractionate the different lipid classes, and after extraction from the silica, each separated class was derivatized and analyzed in terms of fatty acids classes by GC-MS [62,75]. Results of these studies showed that in both *S.*

thermophilus WT and ST6 strains, the fatty acids 14:0, 16:0, C16:1, 18:1 cyc-19cyc are the most abundant, whereas the seven strains of *B. cereus* contained i-15:0, ai-15:0, i16:0, 16:0, i17:0 and a17:0 as the main fatty acyl components of the phospholipids.

I.4.1.3- Analysis of the lipid profile of Gram-positive bacteria by mass spectrometry-based approaches

Mass spectrometry (MS) based approaches have been used for the identification and quantification of lipids in bacteria, and to elucidate taxonomic classification and differentiation. MS can be used to analyze the total lipid extract without previous fractionation, but this has the disadvantage of suppression of ions of lipid classes with lower ionization efficiency. To overcome this, several approaches coupled with MS analysis such as offline with TLC-MS or online with LC (LC-MS) can be used. LC-MS allows the largest coverage of lipid identified and reduces cross suppression following a more sensitive and quantitative detection of minor or poorly ionizing lipid species within complex mixtures.

The identification of lipids by MS has achieved at two different levels of analysis. First, the number of lipid species and their molecular weight is achieved by the information gathered in the MS data, by the identification of the type of ions formed in the source of the mass spectrometry, $[M + H]^+$, $[M + NH_4]^+$, $[M - H]^-$, and identified in the MS spectra. Also, confirmation of the identity of the polar head groups and the composition of fatty acyl chains is achieved by interpretation of MS/MS data. In fact, the MS/MS spectra show specific fragment ions and/ or fragmentation pathways that are specific to the classes of lipids. For example, in the case of, PG, the MS/MS spectra of the $[M + NH_4]^+$ adduct, showed the typical fragmentation pathways due to neutral loss of the polar head group, seen as a mass loss of 159 [48] (Table 2). The MS/MS spectra of the $[M + H]^+$ ions of Lys-PG showed typical neutral loss (NL) of 300 Da and PE showed the NL of 141 Da. Table 2 summarizes the typical fragmentation pathways observed for the phospholipids identified in bacteria. The MS/MS spectra of glycolipids MGDG and DGDG showed characteristic NL of sugar units, -162 Da and also -loss of 2x162 Da (for DGDG) (table 2). The lipoteichoic acid derivatives (LTAP) showed in the MS/MS spectra of the $[M - H]^-$ the product ions 79, 153,

and 171, while the LTAP-Ala showed also the product ion at m/z 88.0, 79.0 and the NL of 89.0 Da [52].

Table I.2 Typical specific fragmentation pathway (product ions and neutral loss (NL)) of polar lipid from Gram-positive bacteria identified in the MS/MS spectrum.

Lipid class	Ionization mode	Product ion	Neutral loss	Reference
PG	$[M- H]^-$	m/z 171		[49]
	$[M + NH_4]^+$	m/z 551.5	- 17 Da	
			- 172 da	
Lys- PG	$[M- H]^-$	m/z 145.1		[66,67]
	$[M+ H]^+$	m/z 301.1	- 300 Da	
PE	$[M- H]^-$	m/z 196.0		[49]
	$[M+ H]^+$	m/z 551.5	- 141 da	
MGDG	$[M + NH_4]^+$	m/z 551.5	- 197 Da	[66]
	$[M + CH_3CO_2]^-$			
	$[M + HCO_2]^-$			
	$[M + Cl]^-$			
DGDG	$[M + NH_4]^+$	m/z 551.5	- 359 Da	[66]
	$[M + CH_3CO_2]^-$			
	$[M + HCO_2]^-$			
	$[M + Cl]^-$			
LTAP	$[M- H]^-$	m/z 79.0		[52]
		m/z 153.0		
		m/z 171.0		
LTAP-Ala	$[M- H]^-$	m/z 88.0	- 89 Da	[52]
		m/z 79.0		
		m/z 153.0		

Recently, MS and LC-MS approaches have been successfully used in the analysis of the lipidome of biological samples and, more recently, for the characterization of bacteria [67,69,85]. MS has received attention for bacterial detection and differentiation due to its capability, when used in conjunction with chromatographic separation, to identify compounds in complex mixtures [19,20]. It has the advantage of analyzing, in only one step, the entire organism's lipidome.

The application of MS to bacterial identification was done initially by direct analysis of the total lipid extract obtained from bacteria using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to attempt to analyze phospholipids in the whole cell bacteria [22,23]. The use of MALDI-MS for phospholipids analysis helped to overcome numerous problems related to the complexity and diversity of extracts, as often encountered in bacteria material [24] (Table 1). With this MALDI-TOF approach, 9PE and 1PG were identified in *E. coli* [49]. Kondakova and co-workers used MALDI-TOF MS imaging coupled to HPTLC to screen of phospholipids classes of *P. fluorescens* MFAF76a and identified 3PG, 4PE and 2PC[38]. Guan and co-workers 2012 studied the lipid compositions of 11 representative strains of *C. botulinum* and a strain of *C. sporogenes* by MS [87]. All strains contained PG, CL, and PE in both the all-acyl and the alk-1'-enyl (plasmalogen) forms and Ala-PG and Lys-PG. *C. butyricum*, *C. beijerinckii* and *C. acetobutylicum* contained lipids characteristic of these saccharolytic species: a glycerol acetal and a PG acetal of the plasmalogen form of PE. *C. perfringens* was analyzed by LC-MS allowing the identification of PG, PE, lysyl-PG, Ala-PG and CL [68,86].

Electrospray ionization mass spectrometry also has been used for the analysis of the total lipid extract as a tool for bacterial identification [25]. It was used for the study of the Gram-positive bacteria *C. botulinum* and allowed the identification of the classes PlaGAPlaE, Lys-PG, Ala-PG. *L. monocytogenes* lipid extract was analyzed by ESI-MS to determinate the fatty acid substituents and their position on the glycerol backbone of the polar lipids, mainly PG, CL, lysyl-CL and DGDG [46]. *L. plantarum* was analyzed by ESI-MS and allowed identification of four major glycolipids of *L. plantarum*: b-D-glucopyranosyl-(1-6)-a-D-galactopyranosyl-(1-2)-a-D-glucopyranosyldiglyceride. The a-D-Glcp-diglyceride, a-D-Galp-(1-2)-a-D-Glcp-diglyceride, b-D-Glcp-(1-6)-a-D-Galp-(1-2)-6-O-acyl-a-D-Glcp diglyceride and the b-D-Glcp-(1-6)-a-D-Galp-(1-2)-a-D-Glcp-diglyceride [82]. In a published work using MS for direct analysis of the total lipid extracts

by MALDI-MS or ESI-MS only few lipid species were identified. More recently the analysis of the total lipid extract by LC-MS has been used with success for the characterization of the lipidome of some bacteria. It allows the identification and quantitation of phospholipid and glycolipids in one single run [21].

I.4.1.4 Lipidomic profile typical of Gram-positive bacteria using LC-MS

Despite the development of the LC-MS-based lipidomics methodologies, there are still few studies on lipid profile analysis of Gram-positive bacteria (Table 1). The few studies published revealed that this approach has the advantage of providing the separation of lipids and analysis with higher sensitivity and more accurate identification. In LC-MS, the identification of each lipid species is achieved by the information of the retention time, MS information and specific identification by tandem mass spectrometry (MS/MS) data analysis and interpretation [26]. The LC-MS analysis of lipids in bacteria was done either using reversed phase as well as normal phase and hydrophilic interaction chromatography (HILIC) columns. Using the reverse phase, the separation of lipid species is based on their hydrophobic components, thus it is dependent on the fatty acid composition. LC-MS using reverse phase have been scarcely used in bacteria and there is only one published work on LC-MS using C18 reverse phase for *B. licheniformis* lipid species [91]. In LC-MS using the normal phase and HILIC columns, lipid species are separated based on the polar head polarity and allows the separation of lipids class. LC-MS for lipidomic studies is widely using HILIC or normal phase columns. In those cases, the separation is based on polarity and provides lipid class-specific separation. It is quite important for the discrimination of the isobaric species, such as the case of isobaric PE and PC species [30]. In LC-MS the identification of lipid species is achieved by the assignment of the retention time, identification of the ions observed in the LC-MS spectra and also by the analysis of the LC-MS /MS data. Overall, it allows identifying the ions attributed to the molecules within each lipid classes, as well as their composition in fatty acyl chains. Quantification of each lipid species can be obtained by integration of peak area of the RIC chromatogram attributed to each lipid species. Different classes of polar lipid have been identified in bacteria and depending on the chemical structure that can be analyzed in positive or negative ion modes.

Some published work reported the analysis of *Bacillus* genus and related genera by lipidomic approach. *B. subtilis* were studied by LC-MS/MS, LIFT-TOF/TOF or DI-MS. These studies allowed the identification of phospholipid classes as 4PG, 7PE, 1lysyl-PG, CL and PA [59], 10PG, 6PE, 2lysyl-PG, and 3DGDG [49] and 8PG, 4PE, 3PS and 6PA [92]. Furthermore, Almasoud and co-workers used MALDI-TOF-MS or LC-MS to study lipid extracted 33 strains from seven bacterial species belonging to the *Bacillus* and *Brevibacillus* genera with the purpose of discrimination of species. Among the species analyzed, the most studied were those of *B. licheniformis* identified only by Lyso-PI [90]. PLs profile of *E. faecalis* and *E. faecium* were characterized by LC-MS and allowed the identification of 17 PGs, 8 LPGs, 23 CL, 3 GPDGDAG, 5 DGDAG, 3 DAGs, and 4 TAGs species [76] and 2 amino-containing phospholipid lysyl-PG, CL, PA and DPDGDAG [93] while in *E. faecium* 2 amino-containing phospholipid lysyl-PG, CL, PA, and DPDGDAG were identified [93]. As for the genus *Staphylococcus*, *S. aureus*, total lipidome was characterized by RP-LC-Q-TOF-MS or HILIC-MS/MS/MS. These studies allowed to identify 7 PG, 7 LPG, 10 CL, 13 DG, 21 DGDG, 8MGDG [77], PG, CL, Lysyl-PG, PC and PA [61]. Conversely, in Gram-positive bacteria and in the genus *Clostridium* plasmalogens with an alk-1-enyl ether substituent together with a normal fatty acid linked at glycerol were identified [78,79]. Among several polar lipids, in *C. novyi* NT through and LC-MS approaches the classes PE, PG, lysyl-PG, alanyl-PG, PS e phosphatidylthreonine (PT) and their plasmalogens were identified, [81].

Overall, the use of mass spectrometry techniques, especially ESI-MS and MALDI-MS, and LC-MS have clearly advanced the field of lipidomics in the profiling of diverse lipids or other metabolites in complex biological samples. MS-based approaches have been used for the identification of polar lipids isolated from the membrane, various phospholipid classes PG, PE, CL, lysyl-PG, and plasmalogen and glycolipids classes DGDG and MGDG that are the main predominant component of Gram-positive bacteria. Identification of the bacteria lipidome will be important because, in bacterial pathogens the cell membrane, is the target of several antibiotics and antimicrobial peptides and, thus, the lipid composition of the membrane is essential for the antibiotic-membrane interaction. In the lipidome, the signature is specific per bacteria and can be used for taxonomic and classification purposes. Furthermore, the identification of specific lipids can propose new biotechnological applications for these bacteria.

I.5 The aim of the work

The lipid profile of *B. licheniformis* I89 is completely unknown. Although several studies report the alteration of the fatty acid profile in the *Bacillus* spp, membrane, very few have focused on the lipidome characterization. In the present study, the profiling of the polar lipidome of *B. licheniformis* I89 was studied for the first time. The work aims at contributing to a better understanding of the adaptation of the lipid metabolism of *Bacillus* under different growth conditions. Herein, gas chromatography and liquid chromatography, mass spectrometry (MS) and tandem mass spectrometry (MS/MS) based approaches were performed to clarify the following aspects:

- identify the fatty acid profile of *B. licheniformis* I89, and evaluate the adaptation of the fatty acid profile to different growth conditions: temperature (37 and 50°C) and stages of cell growth (lag, exponential and stationary);
- characterize the lipidome of *B. licheniformis* I89 at 37 °C and at different stages of growth (lag, exponential and stationary),
- evaluate the effect of the antibiotic vancomycin on the lipid composition of *B. licheniformis* I89 at the different growth stages (lag and exponential) at 37 ° C.

The analytical approach employed, and the main results obtained during the development of this study are described in the following chapters:

Chapter II - Decoding the fatty acid profile of *Bacillus licheniformis* I89 and its adaptation to different growth conditions to investigate possible biotechnological applications

Chapter III - Lipidomic signature of *Bacillus licheniformis* I89 during the different growth phases unravelled by high-resolution liquid chromatography-mass spectrometry

Chapter IV – Changes in *Bacillus licheniformis* I89 membrane lipid composition after exposure to Vancomycin.

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CHAPTER II

II. DECODING THE FATTY ACID PROFILE OF *Bacillus licheniformis* I89 AND ITS ADAPTATION TO DIFFERENT GROWTH CONDITIONS TO INVESTIGATE POSSIBLE BIOTECHNOLOGICAL APPLICATIONS

The results and discussion presented in this section were integrally published as follow:

Celestina Lopes, Joana Barbosa, Elisabete Maciel, Elisabete da Costa, Eliana Alves, Fernando Ricardo, Pedro Domingues, Sónia Mendo, M. Rosário M. Domingues (2019)

Decoding the fatty acid profile of *Bacillus licheniformis* I89 and its adaptation to different growth conditions to investigate possible biotechnological applications, *Lipids*, (2019)

Decoding the Fatty Acid Profile of *Bacillus licheniformis* I89 and Its Adaptation to Different Growth Conditions to Investigate Possible Biotechnological Applications

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Received: 15 August 2018 / Revised: 1 March 2019 / Accepted: 1 March 2019
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Abstract *Bacillus licheniformis* I89 is a Gram-positive bacterium, a producer of the lantibiotic lichenicidin. No information is available on its fatty acid (FA) composition. *Bacillus* species are rich in branched FA (BrFA), claimed to be beneficial to human health and to treat diseases. Herein, the FA profile of *B. licheniformis* I89 was evaluated under different growth conditions: at two growth temperatures (37 and 50 °C) and at different growth phases (lag, exponential, and stationary), using gas chromatography–mass spectrometry. The FA profile revealed predominant BrFA of the iso-series and anteiso-series (i-15:0, ai-15:0, i-16:0, i-17:0, and ai-17:0) and low amounts of saturated FA (14:0, 16:0, and 18:0). Comparing the FA profiles at different temperatures, in the lag phase, at 50 °C, there was a decrease of ai-17:0 and a decrease of i-15:0 in the exponential phase, in comparison with 37 °C. In all growth phases, there was a decrease of ai-15:0 and an increase of i-17:0. From the lag to the stationary phase, at 50 °C, there was a decrease of ai-17:0 and i-16:0, whereas i-15:0 increased, while at 37 °C, there was an increase of i-15:0 and i-16:0, and a decrease in ai-15:0 and ai-17:0. *B. licheniformis* I89 can adapt its FA profile, at moderate temperatures, by changing the iso-FA and anteiso-FA composition and the iso/anteiso ratio. This nonpathogenic

bacterium species can be used as a source of BrFA with putative beneficial health effects for gut protection and with reported antitumor properties, foreseeing its use for producing compounds with biotechnological applications.

Keywords Fatty acid · GC–MS · Gram-positive bacteria · Lipidomics

Lipids (2019).

Abbreviations

BrFA	branched FA
FA	fatty acid
FAME	fatty acid methyl ester
GC–MS	gas chromatography–mass spectrometry
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
OD	optical density
PCO	principal coordinates analysis
PERMANOVA	permutational multivariate ANOVA
SIMPER	similarity percentages

Introduction

Lipids are major components of cell membranes, playing important functions as structural elements, protective components, and as signaling molecules (Madigan et al., 2014; Seltmann and Holst, 2002). Bacterial membranes are composed of phospholipid bilayers with proteins integrated into their lipid matrix. Lipid composition modulates the membrane's features and may influence protein functions (Zhang and Rock, 2008). Most bacterial membrane lipids are glycerophospholipids composed of two fatty acyl

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chains, whose composition has a great influence on the properties of the membrane (de Mendoza, 2014). The ability to adjust lipid composition is important on the regulation of bacterial membrane homeostasis and it is crucial for cell adaptation to different environments and, therefore, to promote bacterial survival. Membrane homeostasis is regulated through fatty acid (FA) synthesis *de novo* (Zhang and Rock, 2008), to maintain properties such as the membrane in a liquid crystalline phase (de Mendoza and Cronan, 1983), and to regulate membrane fluidity and viscosity (Ernst et al., 2016; Zhang and Rock, 2008), which is crucial for its proper function. The most common FA in bacterial lipids have chain lengths between C₁₂ and C₂₀. They can be straight-chain or branched-chain with a methyl group on the *n*-2 or *n*-3 carbons in the chain (iso (i) and anteiso (ai), respectively (Fig. 1). Branched FA (BrFA) are commonly observed in bacteria, namely in *Bacillus*, and their occurrence and distribution is an important biomarker in taxonomy (Kaneda, 1991). Yet, the FA composition may vary among species, depending on the growth conditions.

Bacteria can adapt and grow in environments with different physicochemical conditions such as temperature, salinity, pH, or pressure by modifying their membranes to keep them in a fluid state (Mrozik et al., 2004). It has been reported that temperature influences the FA profile. Bacterial growth at low temperatures stimulates the biosynthesis of short-chain FA, while growth at high temperatures promotes the synthesis of long-chain FA and an increase in the iso-series of BrFA, thus, increasing the iso/anteiso ratio of BrFA (Kaneda, 1977). At high temperatures, there is an increase in saturated and BrFA synthesis, leading to a decrease in the membrane fluidity and better thermostability (Torsvik and Øvreås, 2008). Furthermore, the FA composition of the lipid bilayer influences many essential membrane-associated functions, such as the passive permeability of hydrophobic

molecules, active solute transport, protein–protein interactions, and it promotes alterations in the interaction between proteins and lipids (Zhang and Rock, 2008).

Some studies have already reported fluctuations in the FA profile of some *Bacillus* species, namely *B. subtilis* (Beranová et al., 2008), *B. licheniformis* (El-Hendawy et al., 2012), and *B. simplex*, in which a notable variation was observed in the FA profile in response to growth temperature changes (Sikorski et al., 2008). The FA profile has also been used with taxonomic purposes to differentiate *Bacillus* strains (Diomandé et al., 2015), and to distinguish spores from *B. cereus* T-strain that depended on the composition of the growth medium (Diomandé et al., 2015; Ehrhardt et al., 2010).

Several *Bacillus* species are rich in BrFA, which have been claimed to be beneficial to human health and to treat diseases. These BrFA are also present in food, such as meat and milk, and are considered bioactive compounds, namely to have an important role in the maintenance of gut homeostasis (Astrup, 2014; Chikwanha et al., 2018; Ran-Ressler et al., 2014). Also, free BrFA were tested against cancer cells and have shown antiproliferative properties and the ability to enhance cell death in breast cancer cells, thus being a putative candidate for chemotherapy of cancer (Wongtangtintharn et al., 2004; Yang et al., 2000).

Members of the genus *Bacillus* are producers of compounds with many important biotechnological applications, such as proteases, amylases, surfactants, and lantibiotics (Stein, 2005). Lichenicidin is a two-component lantibiotic that inhibits the growth of relevant pathogens, including the methicillin-resistant *Staphylococcus aureus* (MRSA) and *Listeria monocytogenes* (Caetano et al., 2011). Lichenicidin is produced, among other compounds, by *B. licheniformis* I89 (Caetano et al., 2011; Mendo et al., 2004), a bacterium frequently found in soil. *Bacillus licheniformis* I89 can be used for biotechnological applications, and it can be easily metabolically adapted to different laboratory conditions (Caetano et al., 2015). Also, considering their putative richness in BrFA, as found in other *Bacillus*, characterization of the FA profile of this strain is desirable, foreseeing future applications. Thus, in the present study, the total FA profile of this bacterium was identified and the adaptation of this FA profile was evaluated according to the growth temperature (at 37 and at 50 °C) and at different growth phases (lag, exponential, and stationary phases).

Materials and Methods

Bacteria and Growth Conditions

Bacillus licheniformis I89 was isolated from a hot spring on an island in the Azores and identified by traditional

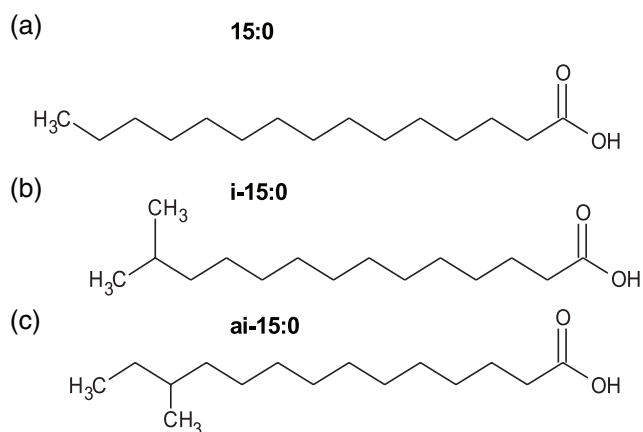


Fig. 1 Main classes of fatty acids identified in *B. licheniformis* I89: (a) saturated fatty acids represented here by 15:0; branched fatty acids represented here by (b) i-15:0, (c) ai-15:0

phenotypic methods (API 50E), phagotyping, and molecular methods (Mendo et al., 2000). Liquid cultures were prepared in M medium containing 10 g/L of NaCl, 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of KH_2PO_4 , with a final pH of 6.5, adjusted with NaOH (Mendo et al., 2004). This medium is deprived of free FA. A preinoculum was prepared to inoculate the medium for total lipid extraction, as follows: a single colony was inoculated in 10 mL of M medium in 50 mL Falcon tubes and the cultures were allowed to grow overnight at 37 °C, at 200 rpm, until the optical density (OD_{600}) reached 0.9. Then, 1 mL was used to inoculate 100 mL of fresh M medium in 500 mL erlenmeyers. Bacterial cells were allowed to grow at 37 and 50 °C at 200 rpm, until they reached the lag phase (after 3 to 4 h incubation), the exponential phase (after 16 h incubation), and the stationary phase (after 24 h incubation). After growth, the cells were harvested at 8000 rpm for 5 min, at room temperature. The supernatants were discarded, and the cellular pellets were stored at −20 °C until lipid extraction. For the different temperatures, the specific growth rate is somehow different and so is the number of cells (Caetano, 2011). These differences are not so evident for lag and exponential phases, but are more dissimilar in the stationary phase (Caetano, 2011). Although the growth yield was slightly different, the same biomass was used for the FA analysis. The procedure was done in triplicate for each growth phase and at each growth temperature.

Lipid Extraction

The bacterial lipids were extracted from the pellets stored at −20 °C, according to Alves et al. (2013). Briefly, 6.5 mL of chloroform/methanol (2:1, by volume) were added to the bacterial cells previously suspended in 2 mL of milli-Q water, in glass centrifuge tubes. The mixture was well homogenized by vigorously inverting the tubes several times and incubated on ice for 210 min. The samples were centrifuged at 568 X *g* for 10 min (Mixtasel, JP Selecta S.A., Barcelona, Spain) at room temperature to separate the phases: an aqueous upper phase and an organic lower phase from which the lipids were obtained. After transferring the organic phase to a clean tube, which dried under a nitrogen stream, the extraction was repeated twice. The extracts were stored in a 2 mL amber glass vial under a nitrogen atmosphere at −20 °C until use.

Quantification of Phospholipids by the Phosphorus Assay

The quantification of phospholipids in the total lipid extracts was performed by measuring the phosphorus amount (Bartlett and Lewis, 1970). Briefly, 250 μL of 70% perchloric acid was added to 10 μL of each sample, which

was previously dried under nitrogen stream, in glass tubes. The samples were incubated at 180 °C for 60 min in a heating block (Block Heater SBH200D/3, Stuart, Bibby Scientific Ltd., Stone, UK), followed by cooling to room temperature. Then, 825 μL of water, 125 μL of 2.5% aqueous solution of ammonium molybdate, and 125 μL of 10% aqueous solution of ascorbic acid were, then, added to the samples, mixing well after each addition. A standard curve of phosphate was prepared, in parallel, using standards from 0.1 to 2 μg of phosphate (standard solution of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 504 mg/L of water, that is, 100 μg of phosphorus mL) and underwent the same treatment as the samples. Samples and standards were, afterward, incubated for 10 min at 100 °C in a water bath (Precistern, JP Selecta S.A., Barcelona, Spain). The absorbance of samples and standards was measured at 797 nm, at room temperature, using a microplate ultraviolet-visible spectrophotometer (Multiskan GO, Thermo Scientific, Hudson, NH, USA).

The relative amount of phospholipid in the samples was estimated by multiplying the amount of phosphorus obtained (in μg), by 25, a conversion factor that multiplied by the mass of phosphorus gives the average mass of a phospholipid.

FA Analysis using Gas Chromatography–Mass Spectrometry (GC–MS)

The total fatty acyl substituents were analyzed after trans-methylation of total lipid extracts (30 μg). The fatty acid methyl esters (FAME) were prepared using a methanolic solution of potassium hydroxide (2.0 M), according to the methodology previously described by Aued-Pimentel et al. (2004). Volumes of 2.0 μL of the hexane solution containing the FAME were subjected to analysis by GC–MS on an Agilent Technologies 6890 N Network (Santa Clara, CA, USA) equipped with a DB-FFAP column with 30 m of length, 0.32 mm of internal diameter, and 0.25 μm of film thickness (J&W Scientific, Folsom, CA, USA). The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range m/z 40–500 in a 1-s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 80 °C, a standing at this temperature for 3 min, a linear increase to 160 °C at 25 °C/min, followed by a linear increase to 190 °C at 2 °C/min. The injector and detector temperatures were 220 and 280 °C, respectively. Helium was used as a carrier gas at a flow rate of 1.4 mL/min. The sum of the areas of the peaks assigned as FA in the chromatogram was considered as the total amount of FA. To determine the relative content of each FA, areas of each individual peak were divided by the sum of the area of all the peaks identified allowing us to determine the relative content of each FA.

Statistical Analysis

Biochemical data are presented as the FA profile, representing the relative abundance of each FA in every growth phase (lag, exponential, or stationary), under two different temperature conditions (37 and 50 °C). Prior to the statistical analysis, the FA data were square root transformed and a similarity matrix was obtained by applying the Bray Curtis coefficient (Anderson, 2008). A permutational multivariate ANOVA (PERMANOVA) was performed to detect significant differences ($p < 0.05$) in the FA profiles of *B. licheniformis* I89 between different temperature conditions. To visualize the differences in the FA profiles of *B. licheniformis* I89 between different temperature conditions, ordination analysis was performed, using principal coordinates analysis (PCO). To test the differences between temperature conditions using each FA individually, a one-way ANOVA was performed, with deviations from normality being tested with the Shapiro test and homogeneity of variance with the Bartlett test (significant differences were defined as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). To describe differences in individual FA and classes of FA between growth phases, similarity percentages (SIMPER) were determined to analyze the percentage contribution of variables to dissimilarities between temperatures (37 and 50 °C). This facilitates the identification of the FA that contributes most to the variations in the assemblage patterns recorded. Only FA that cumulatively contributed to up to 80% of the dissimilarities recorded were selected (Clarke and Gorley, 2006). To evaluate if the FA profiles of *B. licheniformis* I89 showed a consistent

pattern among the three growth stages (lag, exponential, and stationary phases) within temperature conditions (37 and 50 °C), a PERMANOVA was used following an ANOVA. All multivariate statistical tests (PERMANOVA, SIMPER, and PCO) were employed using PRIMER v6 (Clarke and Gorley, 2006) with the add-on PERMANOVA + (Anderson, 2008) while ANOVA were performed using R (R Core Team, 2015).

Results

The FA profile of *B. licheniformis* I89 was determined by GC–MS analysis of FAME from the total lipid extracts, obtained from bacterial cells grown at different temperatures (37 and 50 °C) and at different phases of growth (lag, exponential, and stationary). The FA profile was expressed as relative content, obtained after determination of the peak area of each FA. Thus, quantification was expressed in relative content of the peak area that may not take into account detector and injector discrimination. However, due to the very narrow molecular mass of the FA, the impact of these two issues is minimal.

FA Profile of *B. licheniformis* I89

The identified FA ranged from C₁₄ to C₁₈ and were i-14:0, 14:0, i-15:0, ai-15:0, i-16:0, 16:0, i-17:0, ai-17:0, and 18:0, all of them observed under all the conditions, except for 18:0, which was not present in the stationary phase at 50 °C (Table 1). The FA profile of *B. licheniformis* I89

Table 1 Variation of the fatty acid profiles of *Bacillus licheniformis* I89 in each growth phase, depending on the temperature: lag at 37 °C and at 50 °C, exponential (Exp) at 37 °C and at 50 °C, and stationary (Sta) at 37 °C and at 50 °C and the lag, exponential, and stationary growth phases at 37 °C and at 50 °C

Fatty acids	37 °C			50 °C		
	Lag	Exponential	Stationary	Lag	Exponential	Stationary
i-14:0	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.0	0.7 ± 0.1
i-15:0	27.8 ± 0.1	33.6 ± 0.4	40.0 ± 3.7	25.9 ± 2.6	29.5 ± 0.6	41.4 ± 3.6
ai-15:0	26.6 ± 0.5	22.0 ± 1.4	22.3 ± 0.4	14.7 ± 2.1	15.3 ± 0.3	14.6 ± 0.6
i-16:0	4.8 ± 0.2	6.7 ± 0.6	6.3 ± 0.3	9.6 ± 2.0	8.3 ± 0.8	6.1 ± 0.8
i-17:0	14.6 ± 0.8	16.3 ± 2.5	13.0 ± 2.7	24.5 ± 2.3	22.2 ± 1.8	19.8 ± 2.4
ai-17:0	15.8 ± 0.5	12.1 ± 0.8	9.1 ± 2.2	13.0 ± 1.0	10.8 ± 0.7	8.0 ± 0.7
Branched chain FA	90.3	91.6	91.5	88.3	78.7	90.6
Ratio iso/anteiso	1.1	1.7	1.9	2.2	2.3	3.0
14:0	0.6 ± 0.0	0.6 ± 0.2	0.7 ± 0.2	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.2
16:0	7.5 ± 0.6	6.9 ± 0.7	6.4 ± 1.1	7.7 ± 1.2	8.8 ± 2.2	6.2 ± 2.7
18:0	1.5 ± 0.4	1.2 ± 0.4	1.1 ± 0.4	0.7 ± 0.3	0.5 ± 0.1	
SFA	9.6	8.7	8.2	8.8	9.8	6.5

Fatty acid profiles are expressed as fatty acid relative content (%) calculated by dividing the area of each individual peak assigned for the identified fatty acids, divided by the sum of the area of all the peaks identified. Values are means ± standard deviation. SFA, saturated fatty acid.

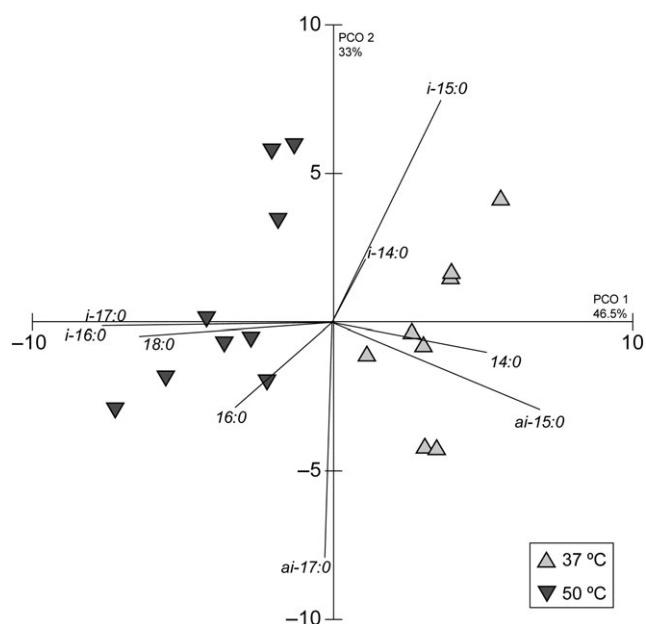


Fig. 2 Principal coordinate analysis of the fatty acid composition of *Bacillus licheniformis* I89 depending on the growth temperature at 37 and at 50 °C

showed to be dependent on the growth temperature (Fig. 2). The significance of these dissimilar profiles was statistically analyzed. When all the FA were considered, PERMANOVA revealed significant differences between FA profiles of *B. licheniformis* I89 grown at two different temperatures ($p = 0.0269$). The SIMPER analysis (Table 2) revealed that 40% of the differences recorded between the FA profile in the different growth temperatures were explained by the FA ai-15:0 and i-17:0. The first two axes of the PCO analysis (PCO1 and PCO2) explain 79.5% of the variation in the FA profiles data set (PCO axis 1: compared the interaction term [temperature \times phases] and revealed significant differences (PERMANOVA, $F = 2.37$ $p = 4.26e-2$). For this reason, the analysis of the FA profile of *B. licheniformis* I89 between temperatures was done separately for each phase, as will be described below.

Table 2 Similarity percentage analysis (SIMPER) identifying which fatty acid contributed to the differences recorded at the growth temperatures of 37 °C and 50 °C in *Bacillus licheniformis* I89

Growth temperature 37 °C and 50 °C		
Fatty acids	Contrib%	Cum.%
ai-15:0	21.7	21.7
i-17:0	19.1	40.8
i-15:0	13.6	54.4
18:0	12.6	67.0
ai-17:0	10.8	77.8
i-16:0	9.29	87.1
16:0	7.58	94.7

FA Profile at Different Growth Temperatures

The FA profile observed at distinct growth phases and growth temperatures is represented in Fig. 3. The most abundant BrFA was i-15:0 at 37 and at 50 °C (Fig. 3), varying significantly at the exponential phase (Fig. 3b). Comparing the FA profile at 37 and at 50 °C, in all growth phases, generally there was an increase in i-16:0 and i-17:0, and a decrease in ai-15:0 and ai-17:0 (Fig. 3). The FA ai-15:0 and i-17:0 changed significantly between temperatures and in all

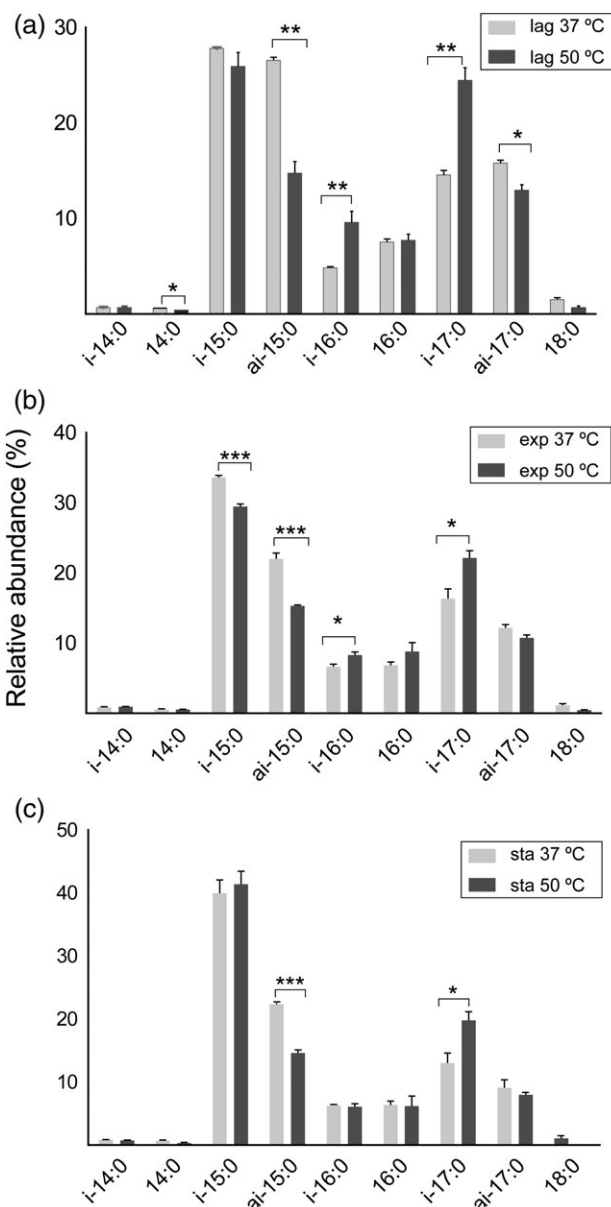


Fig. 3 Comparison of the variation of fatty acid profiles of *Bacillus licheniformis* I89 in each growth phase, depending on the growth temperature: lag at 37 °C and at 50 °C (a), exponential (Exp) at 37 °C and at 50 °C (b) and stationary (Sta) 37 °C and at 50 °C (c). Values are means \pm standard deviation. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

growth phases, i-16:0 showed significant differences only in the lag and exponential phases (Fig. 3a, b), and ai-17 showed a significant difference at the lag phase (Fig. 3a).

In both lag and exponential phases, i-15:0 at 37 °C was slightly higher than at 50 °C. This variation in the BrFA profile at 37 °C and at 50 °C was also assigned to the high increase of the iso/anteiso ratio, which doubled when comparing the same growth phase at two different temperatures (Table 1). At 37 °C, the iso/anteiso ratio increased from the lag to the stationary phase, with values of 1.1, 1.7, and 1.9 for lag, exponential, and stationary phase, respectively. At 50 °C, the ratio is much higher, also increasing from lag to stationary phases, being 2.2, 2.3, and 3.0, respectively.

Palmitic acid (16:0) was the predominant saturated FA at both temperatures 6.9–7.6%, followed by stearic acid (18:0) 0.6–1.3% and myristic acid (14:0) 0.4–0.6% (Fig. 2). The FA 14:0 decreased in the lag phase when cells grew at 50 °C.

FA Profile at Different Growth Phases

The variation of the FA profile in the three growth phases (lag, exponential, and stationary) was evaluated at 37 °C and at 50 °C (Fig. 3a, b, respectively). For all the growth phases and at 37 °C, the most abundant FA was i-15:0, followed by ai-15:0, i-17:0 and ai-17:0. The amount of i-15:0 was significantly different among the growth phases (Fig. 4). Significant differences were observed for ai-15:0, i-16:0, and ai-17:0. The FA ai-15:0 and ai-17:0 between the lag phase and the other growth phases showed a gradual decrease from the lag to the stationary phase, while i-16:0 increases from the lag to the exponential phase. The iso/anteiso ratio increased from the lag to the stationary phase, at 37 °C.

At 50 °C and for all the growth phases, the most abundant FA was i-15:0, followed by i-17:0, ai-15:0, and ai-17:0. i-15:0 increases from the lag to the stationary phase and is significantly different between the lag and the exponential phase and between the lag and the stationary phase, whereas ai-17:0 decreases from the lag to the stationary phases and was significantly different among all growth phases (Fig. 4b). Significant differences were also observed for i-14:0 and 14:0, which slightly increased between the lag and the exponential phases, while i-16:0 differed significantly between the lag and the stationary phase. The FA 16:0 differed significantly between the exponential and the stationary phase. Interestingly, the iso/anteiso ratio increased from the lag to the stationary phase, at 50 °C.

Discussion

In the present study, the FA profile of *B. licheniformis* I89 was investigated for the first time. The FA composition of

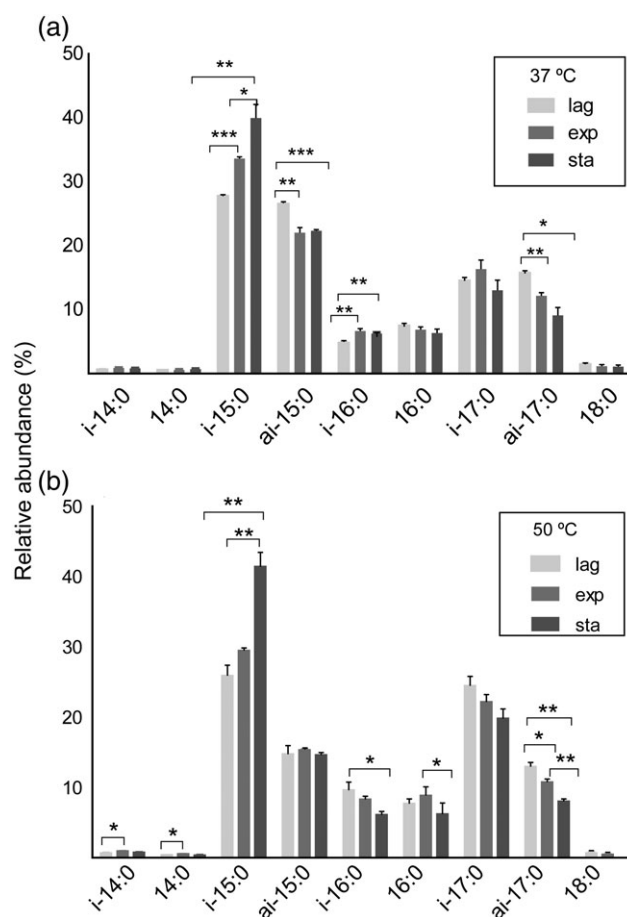


Fig. 4 Comparison of the fatty acid profiles of *Bacillus licheniformis* I89 between the lag, exponential (Exp), and stationary (Sta) growth phases at 37 °C (a) and at 50 °C (b). Values are means \pm standard deviation, * p < 0.05, ** p < 0.01

this strain is very similar to that previously reported for the genus *Bacillus* (Beranová et al., 2008; Chen et al., 2015; Sikorski et al., 2008), namely *B. subtilis* (Beranová et al., 2008), *B. vanillea* (Chen et al., 2015), and *B. megaterium* (Koga, 2012), but somehow dissimilar to the FA profile of *Bacillus* identified in spores and vegetative cells of *B. licheniformis* (Martin and Swenson, 1976). The FA profile of this genus predominantly includes BrFA (iso and anteiso), where i-15:0, ai-15:0, i-16:0, i-17:0, and ai-17:0 represent the main FA (Beranová et al., 2008; Chen et al., 2015; Kämpfer, 1994), even when cells grow at different temperatures (Beranová et al., 2008; Diomandé et al., 2015; Yi et al., 2016). These findings agree with the results obtained in this study. However, El-Hendawy et al. (2012) reported that FA of *B. licheniformis* grown at different temperatures (20, 35, and 50 °C) contained saturated straight-chain FA (9:0, 10:0, 12:0, 13:0, 14:0, 16:0, 17:0, and 20:0) and unsaturated FA (16:1, 17:1, 18:2, and 18:3) (El-Hendawy et al., 2012). Martin and Swenson (1976) found that vegetative cells of *B. licheniformis*, grown at 37 °C,

contained nine FA, including saturated straight chain FA (10:0, 12:0, 15:0, 16:0, and 18:0) and unsaturated chain FA (16:1, 18:1, 18:2, and 18:3). This information contrasts with the results obtained herein and also in what has been reported for *Bacillus*. This dissimilar information can, though, be due to misidentification, because authors used GC-FID for profiling the FA, which is based only on retention times, while in the present work, GC-MS was used, giving a more accurate FA assignment by the identification of the FA based on retention times and interpretation of the mass spectra.

Spores of *B. licheniformis* grown at 37 °C contained 11 FA distributed by saturated straight chain FA (14:0, 15:0, 16:0, 17:0, and 18:0), unsaturated chain FA (16:1, 18:1, and 18:2) and BrFA (i-15:0, i-17:0, and ai-17:0) (Martin and Swenson, 1976). The results obtained herein showed that the FA profile of *B. licheniformis* I89 depends on the growth temperature. Overall, it was observed that, at the higher temperature (50 °C), there is a decrease of ai-15:0 and ai-17:0 and an increase of i-17:0. An increase of i-16:0 was also observed in the lag and in the exponential phases. These results are corroborated by the increase of the iso/anteiso ratio from the lag to the stationary phases and with the growth temperature (at 37 °C and at 50 °C).

Bacillus species can modulate their membrane lipids to maintain membrane fluidity and transport functions in response to external factors (Annous et al., 1997) and pH (Giotis et al., 2007). Such abilities seem to be related to their unusually high iso and anteiso, odd-numbered BrFA content and their ability to regulate the content in BrFA, straight-chain FA, and unsaturated FA (Kaneda, 1977, 1991). It has been reported that iso-FA increase with the increase in the growth temperature, similar to what was observed in the present study. The opposite may also occur, iso-FA may increase at low growth temperature (Koga, 2012; Sikorski et al., 2008).

In *B. subtilis*, grown at various temperatures, between 13 and 50 °C (lowest and highest growth temperature boundaries, respectively), the membrane adaptation to external temperatures afforded a higher content of anteiso-branched rather than iso-branched lipids in lower temperatures (Van De Vossenberg et al., 1999), which corroborates the present results. *B. megaterium* contained i-15:0 (25% of total FA) and ai-15:0 FA (50% of total FA) at 20 °C and 35% i-15:0 and 15% ai-15:0 at 60 °C (Koga, 2012). *B. subtilis* was cultured at optimum (40 °C) and low (20 °C) growth temperatures, but the amount of BrFA was related to the composition of the growth media, where an increase in ai-15:0 and ai-17:0, as well as a decrease of i-15:0 and i-17:0 were observed at 20 °C (Beranová et al., 2008). Overall, the increased relative amount of i-15:0 and i-17:0, and a decrease of the anteiso-homologues with temperature would result in an increase of the melting point

that may lead to a decrease of the membrane fluidity. Thus, these changes might cause the membrane to become less fluid, which would be advantageous as temperature increases (Nordström, 1992). Membrane fluidity is affected by the composition of the BrFA due to the disruptive effect of the methyl group on acyl-chain packing (Zhang and Rock, 2008). The membrane structure is more fluid when the amount of anteiso-FA is higher than the amount of iso-FA due to the fact that the methyl-branch is further away from the end of the FA (de Mendoza, 2014). Several bacteria modify the biosynthesis of these FA to alter their iso/anteiso ratio in response to temperature and pH stress (Zhang and Rock, 2008). Herein, the iso/anteiso ratio as well as the BrFA increase with temperature. Generally, the most abundant saturated FA, 16:0, increases at a higher temperature (Brooke and Hopkins, 2009), but in the present study only a slight increase of this FA was observed at the higher temperature, 50 °C.

The same FA were identified along the lag, exponential, and stationary growth phases. However, the relative abundance of some of these FA varied in each growth phase. An increase was observed in the i-15:0 FA and a decrease in ai-15:0 and ai-17:0, when comparing the lag phase with the exponential phase, and the lag phase with the stationary phase. Interestingly, the iso/anteiso ratio increases from the lag to stationary growth phase at both 37 and 50 °C. Changes in the lipid content during bacterial growth phases have been scarcely reported so far. A single study of *Bacillus* in exponential and stationary phases showed differences in the phospholipids, but the FA profile was not analyzed (Gidden et al., 2009). It has been reported that, frequently, and in most bacterial species, the entrance in the stationary phase leads to structural and physiological changes that result in an increased resistance to stress (Zhang and Rock, 2008). The changes related to the growth phases could afford a less fluid membrane to the cells, which become more fragile as growth proceeds (Nordström, 1992). This can be essential for the modulation of bacterial metabolism and significant to its survival or death.

Also, the knowledge of the modulation of the FA composition in *Bacillus* by the growth conditions is quite important considering that BrFA have been associated with promising biotechnological applications. For instance, the iso-15:0 FA has been described as a potential anticancer agent, by inhibiting proliferation and inducing apoptosis in different cancer cells (Cai et al., 2013; Henshall et al., 2002; Wongtangtharn et al., 2004; Yang et al., 2000; Yu et al., 2014). Also, BrFA can display bioactive properties and gut protection, as reported for BrFA in dairy products and beef (Ran-Ressler et al., 2014). The obtained results indicate that *B. licheniformis* bacterial strains could be a hopeful source of bioactive lipids. Different proportions of BrFA can be important modulators of bacterial membranes

and can play a relevant role in antibiotic resistance. Furthermore, this *Bacillus* species can be a source of BrFA with putative beneficial health effects and therapeutic applications, because they have a beneficial role against inflammation (Ran-Ressler et al., 2014) and antitumor properties (Wongtangtintharn et al., 2004; Yang et al., 2000). They might also be promoters of well-being, because they have shown to be gut protectors (Astrup, 2014; Chikwanha et al., 2018). These properties of BrFA, which were identified in *B. licheniformis* I89, foster the use of this nonpathogenic bacterium for producing compounds with biotechnological applications. Some bacteria are recognized as important producers of compounds with biotechnological applications, because they are easily reproduced on a large scale. They have also been proposed as producers of other interesting lipids, such as for biofuels (Bentley et al., 2016; Tao et al., 2015), and most importantly, as producers of compounds with health benefits.

In conclusion, in the present study, the FA profile of *B. licheniformis* I89 was analyzed using an approach based on gas chromatography coupled to mass spectrometry. The iso-BrFA and anteiso-BrFA are the most abundant of the total FA. The results showed that: i) there is an increase in the iso/anteiso FA ratio with temperature, which reveals the predominance of iso-branched chains and anteiso-branched-chains of FA; and ii) temperature affects the bacterial FA composition. Accordingly, at 37 °C the membrane of *B. licheniformis* I89 showed the presence of a higher amount of ai-17:0 FA and less i-15:0. Also, it was noticed a high amount of ai-17:0 in the lag phase and a reduction of iso-15:0. Understanding changes in the FA composition of *B. licheniformis* I89 and its membrane homeostasis, under different growth conditions, can be important to comprehend the adaptation mechanism of this bacterium because it is a strain with relevant biotechnological applications, and these fluctuations can interfere with the yield of the products it synthesizes.

Acknowledgements Thanks are due to the University of Aveiro, Fundação para a Ciência e a Tecnologia (Fundação para a Ciência e a Tecnologia (FCT)) and Ministério da Educação e Ciência, QREN, COMPETE for the financial support to the Organic Chemistry, Natural and Agrofood Products (QOPNA) research Unit (FCT UID/UI/00062/2013) and Centre for Environmental and Marine Studies (CESAM) (UID/AMB/50017/2013), through national funds and where applicable cofinanced by the FEDER, within the PT2020 Partnership Agreement, and also to the Portuguese Mass Spectrometry Network (REDE/1504/REM/2005). C.L. is grateful to Calouste Gulbenkian Foundation for her PhD grant (process number 135624). J.B. (SFRH/BD/97099/2013), E.M. (SFRH/BPD/104165/2014), E.C. (SFRH/BD/52499/2014), E.A. (SFRH/BPD/109323/2015), and F.R. (SFRH/BD/84263/2012) are grateful to FCT for their grants.

Conflict of Interest The authors declare that they have no conflict of interest.

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CHAPTER III

III. LIPIDOMIC SIGNATURE OF *Bacillus licheniformis* I89 DURING THE DIFFERENT GROWTH PHASES UNRAVELLED BY HIGH-RESOLUTION LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

The results and discussion presented in this section were integrally published as follow:

Celestina Lopes, Joana Barbosa, Elisabete Maciel, Elisabete da Costa, Eliana Alves, Pedro Domingues, Sónia Mendo, M. Rosário M. Domingues (2019)

Lipidomic signature of *Bacillus licheniformis* I89 during the different growth phases unraveled by high-resolution liquid chromatography-mass spectrometry, Archives of Biochemistry and Biophysics, 663:83-94



Lipidomic signature of *Bacillus licheniformis* I89 during the different growth phases unravelled by high-resolution liquid chromatography-mass spectrometry

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ARTICLE INFO

Keywords:

Phospholipid
Glycolipid
Mass spectrometry
Lipidomics
Gram-positive bacteria

ABSTRACT

Bacillus licheniformis I89 is a non-pathogenic, Gram-positive bacterium, frequently found in soil. It has several biotechnological applications as producer of valuable compounds such as proteases, amylases, surfactants, and antibiotics. Herein, it is reported the identification of the polar lipidome of *B. licheniformis* I89 during the different growth phases (lag, exponential and stationary) at 37 °C. The analytical approach relied on hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometry (HILIC-ESI-MS), accurate mass measurements and tandem mass spectrometry (MS/MS). In the lipidome of *B. licheniformis* I89 were identified four phospholipid classes: phosphatidylethanolamine, phosphatidylglycerol, lysyl-phosphatidylglycerol, and cardiolipin; two glycolipid classes: monoglycosyldiacylglycerol and diglycosyldiacylglycerol; and two phosphoglyceroglycolipid classes: mono-alanylated lipoteichoic acid primer and lipoteichoic acid primer. The same lipid species were identified at the different growth phases, but there were significant differences on the relative abundance of some molecular species. There was a significant increase in the 30:0 lipid species and a significant decrease in the 32:0 lipid species, between exponential and stationary phases, when compared to lag phase. No differences were observed between exponential and stationary phases. The lipidomic-based approach used herein is a very promising tool to be employed in the study of bacterial lipid composition, which is a requirement to understand its metabolism and response to growth conditions.

1. Introduction

Bacterial membranes are composed mainly by glycerolipids such as phospholipids (PL) and glycolipids (GL) which have an important role in membrane properties and function and are the main lipid players in signalling and regulation events in these organisms [1]. The regulation and homeostasis of membrane lipids is essential to the bacterial growth, differentiation, viability and proliferation [1,2]. Furthermore, lipid metabolism is involved in biological membrane synthesis and energy homeostasis during pathogen replication and resistance [3], since both bacterial lipid composition and lipid organization into domains are important for signalling, secretion, normal physiology, virulence and antibiotic resistance [1]. Despite the importance of lipids in bacterial membranes, many studies focus only on the fatty acid (FA) composition

of the membrane of bacteria [4–6]. However, most of the FA in bacteria are esterified to other lipids, namely polar lipids, as PL and GL, that have been mostly overlooked.

Only a few studies reported the lipidome of bacteria. This is possibly due to complexity and distinct types of polar lipids that can be found in different bacteria [7]. Also, the most common analytical methods to study lipids in bacteria, such as thin-layer chromatography (reviewed by Ref. [8]), nuclear magnetic resonance [9,10], and gas chromatography (GC) [11], provide limited information. More recently, mass spectrometry (MS)-based approaches have been used for the detailed analysis of the membranes lipidome [12]. These approaches include the direct analysis of the lipid extracts by electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) or liquid chromatography (LC) coupled to MS. These lipidomic LC-MS-based approaches

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<https://doi.org/10.1016/j.abbi.2018.12.024>

Received 17 October 2018; Received in revised form 4 December 2018; Accepted 21 December 2018

Available online 23 December 2018

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have been successfully used to identify a high number of lipids in both Gram-positive and Gram-negative bacteria [13–16].

Direct analysis by ESI-MS of the total lipid extracts of the Gram-positive bacterium *Listeria monocytogenes* identified phosphatidylglycerols (PG), cardiolipins (CL), lysyl-cardiolipins (lys-CL), and diglycosyldiacylglycerols (DGDG) [16]. DGDG and monoglycosyldiacylglycerols (MGDG) were identified in *Streptococcus pneumoniae* [17], and phosphatidic acid (PA), phosphatidylethanolamines (PE), PG and phosphatidylserines (PS) in *Bacillus subtilis* SDB206 [18]. MALDI-MS was also used to study the lipid composition of *B. subtilis* which allowed for the identification of PL and GL classes (PG, PE, lys-PG and DGDG) [19]. An LC-MS-based approach was used for characterizing the lipidome of Gram-positive bacteria including *B. subtilis* [15,18], *Staphylococcus* [14,20] and *Clostridium* [21,22]. LC-MS was used to taxonomically discriminate bacteria from different strains of *Bacillus* and *Brevibacillus*, showing that this can be a promising tool for bacteria classification [23]. Nonetheless, only a few species of PE, lyso-phosphatidylinositol (lyso-PI), and PA were identified [23]. Hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometry (HILIC-ESI-MS) was used to profile the phospholipidome of *Staphylococcus warneri* [14] but, so far, there are no studies using HILIC-ESI-MS in the lipidome analysis of *Bacillus* species.

Bacillus licheniformis is a Gram-positive, endospore-forming bacterium, a non-pathogenic member of the genus *Bacillus*, that belongs to the *B. subtilis* group. It is commonly found in soil and has many biotechnological applications. It produces valuable compounds, such as proteases, amylases, surfactants, immunosuppressors, antimicrobials (e.g., lichenicidin, bacitracin, surfactin), lipids, among others [24,25]. *B. licheniformis* I89 has been described as a lantibiotic (lichenicidin) producer [25]. Lanthipeptides (lanthionine-containing peptides) are ribosomally synthesized and posttranslationally modified peptides (RiPPs). These are natural products with diverse biological activities, namely antibacterial activity [26]. Considering the potential biotechnological applications of *B. licheniformis* I89, the lipidome of this bacterial strain was characterized by HILIC-ESI-MS at the different growth phases (lag, exponential and stationary) at 37 °C.

2. Materials and methods

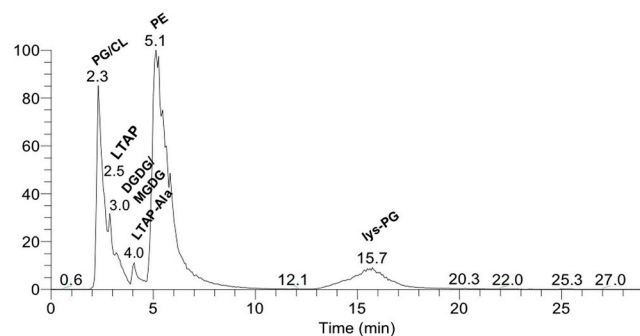
2.1. Bacteria and growth conditions

B. licheniformis I89 was isolated from a hot spring environment from the Azores islands [27]. Liquid cultures were prepared in M medium: 10 g L⁻¹ of NaCl, 10 g L⁻¹ of tryptone, 5 g L⁻¹ of yeast extract, 10 g L⁻¹ of KH₂PO₄, with a final pH of 6.5, adjusted with NaOH [28]. An overnight pre-inoculum was prepared to inoculate the medium for total lipid extraction, as follows: a single colony was inoculated in 10 mL of M medium in a 50 mL falcon tube, the cultures were allowed to grow overnight at 37 °C, at 200 rpm, until the OD₆₀₀ reached 0.9. Then, 1 mL of this culture was used to inoculate 100 mL of fresh M medium, in 500 mL Erlenmeyers. Bacterial cells were allowed to grow at 37 °C at 200 rpm, until they reached the lag phase (3–4 h incubation, OD₆₀₀ 0.5), the exponential phase (16 h incubation, OD 1.7–2.0) and the stationary phase (24 h incubation, OD 1.9–2.5). After growth, the cells were harvested at 8000 rpm for 5 min, at room temperature. The supernatants were discarded, and the cellular pellets were stored at –20 °C until further use. The procedure was done in triplicate for each growth phase at 37 °C.

2.2. Lipid extraction

The total bacterial lipids were extracted from the pellets previously stored at –20 °C, as described in Alves and co-workers (2013) [14]. Briefly, 6.5 mL of chloroform/methanol (2:1, by volume) were added to the bacterial cells previously suspended in 2 mL of milli-Q water, in glass centrifuge tubes. The mixture was well homogenized by inverting

a) HILIC-ESI-MS – Negative ion mode



b) HILIC-ESI-MS – Positive ion mode

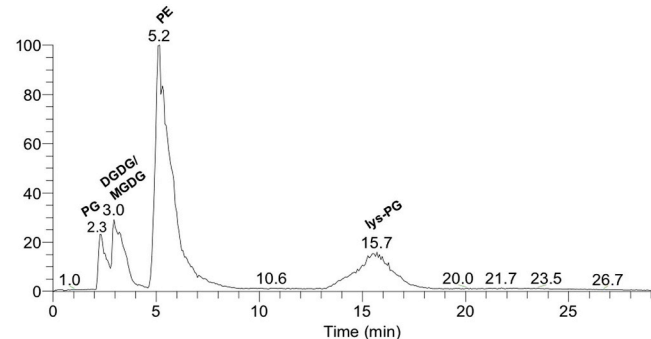


Fig. 1. HILIC-ESI-MS chromatograms of total lipid extracts of *B. licheniformis* I89 in (a) negative ion mode and (b) positive ion mode and the retention time (RT) of each polar lipid class. PG: Phosphatidylglycerol, RT 2.3 min; CL: Cardiolipin, RT 2.3 min; LTAP: Lipoteichoic acid primer, RT 2.5 min; DGDG: Diglycosyldiacylglycerol, RT 3.0 min; MGDG: Monoglycosyldiacylglycerol, RT 3.0 min; LTAP-Ala: Mono-alanylated lipoteichoic acid primer, RT 4.0 min; PE: Phosphatidylethanolamine, RT 5.1 min; lys-PG: lysyl-phosphatidylglycerol, RT 15.7 min.

vigorously the tubes several times and incubated on ice for 210 min. The samples were centrifuged at 568 x g for 10 min (Mixtasel, JP Selecta S.A., Barcelona, Spain) at room temperature to separate the phases: the aqueous (upper) phase and the organic (lower) phase from which the lipids were obtained. After transferring the organic phase to a clean tube, the extraction was repeated twice from the tube containing the bacterial pellet. The extracts were dried under a nitrogen stream, dissolved in chloroform, transferred to 2 mL amber glass vials and stored under a nitrogen atmosphere at –20 °C until use.

2.3. Quantification of phospholipids by phosphorus assay

The quantification of PL was performed by measuring the phosphorus amount in the total lipid extracts (adapted from Ref. [29]). Briefly, lipid hydrolysis was performed by adding 125 µL of 70% perchloric acid to the samples and phosphate standards (100 µg mL⁻¹ of sodium phosphate dibasic dihydrate, ranging from 0.10 to 2.00 µg of phosphorus) in glass tubes. The samples incubated 60 min at 180 °C in a heating block (Block Heater SBH200D/3, Stuart, Bibby Scientific Ltd., Stone, UK), and cooled down to room temperature. Milli-Q water (825 µL), ammonium molybdate (125 µL, 25 g L⁻¹ in water), and ascorbic acid (125 µL, 100 g L⁻¹ in water) were then added to the samples and standards, homogenizing well between each addition. Samples and standards were, then, incubated for 10 min at 100 °C in a water bath (Precistern, JP Selecta S.A., Barcelona, Spain). The absorbance of standards and samples was measured at 797 nm, at room temperature, in a microplate UV-Vis spectrophotometer (Multiskan GO, Thermo Scientific, Hudson, NH, USA).

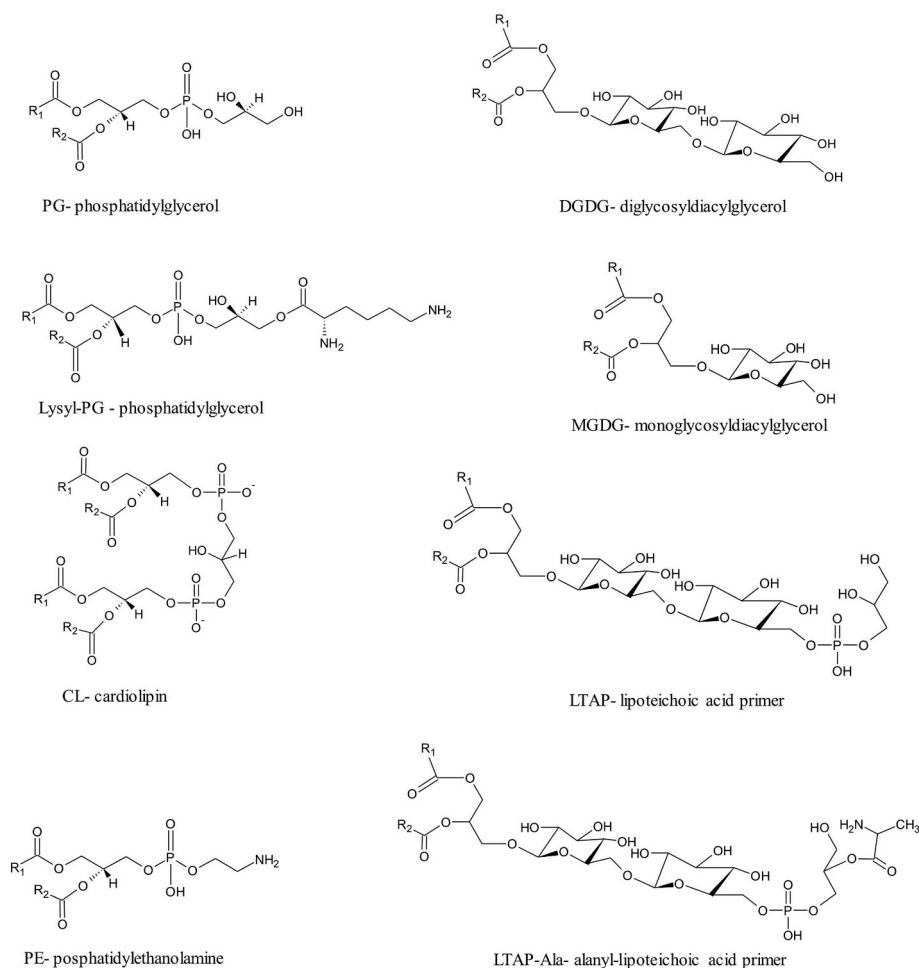


Fig. 2. Chemical structures of the polar lipids identified in *B. licheniformis* I89.

2.4. Hydrophilic interaction liquid chromatography - electrospray ionization - mass spectrometry (HILIC-ESI-MS)

Polar lipids were analyzed by HILIC-ESI-MS on a Thermo Scientific Accela™ HPLC system with an autosampler online coupled to a Q-Exactive™ mass spectrometer with Orbitrap® technology (Thermo Fisher Scientific, Bremen, Germany). The solvent system consisted of two mobile phases: mobile phase A was acetonitrile/methanol/water, 50:25:25 per volume, with 1 mM ammonium acetate, and mobile phase B was acetonitrile/methanol, 60:40 per volume, with 1 mM ammonium acetate. Initially, 0% of mobile phase A was held isocratically for 8 min, followed by a linear increase to 60% of A within 7 min and a maintenance period of 15 min, returning to the initial conditions in 10 min. A volume of 5 μ L of each sample containing 5 μ g of lipid extract and 95 μ L of mobile phase B was introduced into the Ascentis® Si column (15 cm \times 1 mm, 3 μ m, Sigma-Aldrich) with a flow rate of 40 μ L min⁻¹ and at 30 °C. The mass spectrometer was operated simultaneously in positive (electrospray voltage 3.0 kV) and negative (electrospray voltage -2.7 kV) ion modes, with a resolution of 70 000 (FWHM) and automatic gain control (AGC) target of 1×10^6 . The capillary temperature was 250 °C and the sheath gas flow was 15 U. In MS/MS experiments, a resolution of 17 500 and AGC target of 1×10^5 were used. The cycles consisted in one full scan mass spectrum and ten data-dependent MS/MS scans and were repeated continuously throughout the experiments with the dynamic exclusion of 60 s and intensity threshold of 1×10^4 . Normalized collision energy™ (CE) ranged between 25, 30 and 35 eV. Data acquisition was carried out using the Xcalibur data system (V3.3, Thermo Fisher Scientific, USA). Three bacterial cultures

were analyzed independently for each of the three growth phases. The identification of molecular species of polar lipids was based on the assignment of the molecular ions observed in LC-MS spectra and by the identification of the fragmentation pattern of each class observed in the MS/MS spectrum of each ion [30]. To confirm the identification of molecular species, mass accuracy (Qual Browser) was determined with ≤ 5 ppm.

2.5. Data and statistical analysis

The raw data were processed using the MZmine software 2.32 [31]. First, the mass list was filtered, followed by peak detection and peak processing. During the processing of the raw data, acquired in full MS mode, only peaks with raw intensity upper than $1e4$ and with mass tolerance of 5 ppm were considered. Peak assignment and ion identification based on mass accuracy were performed against an in-house database. Data integration was expressed by the changes of the relative abundance of molecular species of all classes. Variation in the lipidome of *B. licheniformis* I89 was measured in triplicate in three different conditions (lag, exponential and stationary phases). Results were expressed as mean \pm SD using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests to compare the growth phases, after checking for assumptions. Significant differences were determined in relative percentages of molecular species per class ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$). Statistical analysis was performed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

Table 1Molecular species of phospholipids from *B. licheniformis* I89 identified by LC-MS in positive and negative ion modes.

Mass spectrometry data				
Lipid Group	Observed m/z value	Calculated m/z value	Mass deviation [ppm]	Fatty acid chains
Phosphatidylglycerols [M – H] [–]				
PG (28:0)	665.4394	665.4394	0.0556	13:0/15:0; 14:0/14:0
PG (29:0)	679.4543	679.4550	–1.0494	15:0/14:0; 13:0/16:0
PG (30:0)	693.4703	693.4707	–0.5235	15:0/15:0; 14:0/16:0
PG (31:1)	705.4687	705.4707	–2.7825	15:0/16:1
PG (31:0)	707.4860	707.4863	–0.4424	15:0/16:0; 14:0/17:0
PG (32:1)	719.4844	719.4863	–2.6588	15:0/17:1; 15:1/17:0; 16:1/16:0
PG (32:0)	721.5019	721.5020	–0.0859	15:0/17:0; 16:0/16:0
PG (33:0)	735.5170	735.5176	–0.8334	18:0/15:0; 16:0/17:0; 19:0/14:0
PG (34:1)	747.5140	747.5176	–4.8333	16:1/18:0; 17:1/17:0; 16:0/18:1
PG (34:0)	749.5327	749.5333	–0.7511	19:0/15:0; 17:0/17:0
PG (35:0)	763.5484	763.5489	–0.6719	18:0/17:0; 15:0/20:0
Lysyl phosphatidylglycerols [M – H] [–]				
lys-PG (29:0)	807.5502	807.5500	0.2774	14:0/15:0
lys-PG (30:0)	821.5659	821.5656	0.3335	15:0/15:0; 14:0/16:0
lys-PG (31:1)	833.5662	833.5656	0.6886	15:0/16:1
lys-PG (31:0)	835.5812	835.5813	–0.0910	15:0/16:0; 17:0/14:0
lys-PG (32:1)	847.5810	847.5813	–0.3256	15:0/17:1
lys-PG (32:0)	849.5965	849.5969	–0.5002	15:0/17:0; 16:0/16:0
lys-PG (33:0)	863.6123	863.6126	–0.3196	16:0/17:0; 15:0/18:0
lys-PG (34:0)	877.6277	877.6282	–0.5993	17:0/17:0; 15:0/19:0
Phosphatidylethanolamines [M – H] [–]				
PE (28:0)	634.4456	634.4448	1.2893	15:0/13:0; 14:0/14:0
PE (29:0)	648.4606	648.4604	0.2591	15:0/14:0
PE (30:1)	660.4609	660.4604	0.7086	15:1/15:0; 14:0/16:1; 14:1/16:0
PE (30:0)	662.4766	662.4761	0.7819	15:0/15:0; 14:0/16:0
PE (31:1)	674.4759	674.4761	–0.2698	15:0/16:1
PE (31:0)	676.4916	676.4917	–0.1951	15:0/16:0; 14:0/17:0
PE (32:1)	688.4918	688.4917	0.0988	15:0/17:1; 16:1/16:0
PE (32:0)	690.5073	690.5074	–0.1188	15:0/17:0; 16:0/16:0
PE (33:2)	700.4920	700.4917	0.3826	15:0/18:2
PE (33:1)	702.5063	702.5074	–1.5402	15:0/18:1; 16:0/17:1; 17:0/16:1
PE (33:0)	704.5224	704.5230	–0.8971	15:0/18:0; 17:0/16:0
PE (34:1)	716.5206	716.5230	–3.3942	18:1/16:0; 16:1/18:0
PE (34:0)	718.5385	718.5387	–0.2533	17:0/17:0; 15:0/19:0; 16:0/18:0
Cardiolipins [M – H] [–]				
CL (60:0)	1295.8987	1295.9018	–2.3968	15:0/15:0/15:0/15:0
CL (61:0)	1309.9151	1309.9175	–1.7986	15:0/15:0/16:0/15:0
CL (62:0)	1323.9309	1323.9331	–1.6662	15:0/15:0/15:0/17:0; 15:0/16:0/15:0/16:0; 14:0/17:0/16:0/15:0
CL (63:0)	1337.9466	1337.9488	–1.6114	15:0/15:0/16:0/17:0
CL (64:0)	1351.9622	1351.9644	–1.6317	17:0/17:0/17:0/17:0
Cardiolipins [M – 2H] ^{2–}				
CL (30:0)	647.4475			15:0/15:0; 14:0/16
CL (32:0)	661.4632			15:0/15:0; 15:0/17:0
CL (33:0)	667.9647			15:0/15:0; 16:0/17:0
CL (34:0)	675.4785			17:0/17:0; 15:0/19:0; 16:0/18:0
Phosphatidylglycerols [M + NH ₄] ⁺				
PG (30:0)	712.5103	712.5123	–2.8070	15:0/15:0
PG (31:0)	726.5256	726.5285	–4.0081	15:0/16:0
PG (32:0)	740.5412	740.5436	–3.2409	15:0/17:0
Phosphatidylethanolamines [M + H] ⁺				
PE (28:0)	636.4575	636.4604	–4.6240	15:0/13:0; 14:0/14:0
PE (29:0)	650.4742	650.4761	–2.8933	15:0/14:0
PE (30:1)	662.4766	662.4761	0.7819	15:1/15:0; 14:0/16:1; 14:1/16:0
PE (30:0)	664.4901	664.4917	–2.4560	15:0/15:0; 14:0/16:0
PE (31:1)	676.4917	676.4917	–0.0473	15:0/16:1
PE (31:0)	678.5055	678.5074	–2.7737	15:0/16:0; 14:0/17:0
PE (32:1)	690.5074	690.5074	0.0261	15:0/17:1; 16:1/16:0
PE (32:0)	692.5208	692.5230	–3.2230	15:0/17:0; 16:0/16:0
PE (33:2)	702.5063	702.5074	–1.5402	15:0/18:2

(continued on next page)

Table 1 (continued)

Mass spectrometry data				
Lipid Group	Observed m/z value	Calculated m/z value	Mass deviation [ppm]	Fatty acid chains
PE (33:1)	704.5225	704.5230	−0.7551	15:0/18:1; 16:0/17:1; 17:0/16:1
PE (33:0)	706.5362	706.5387	−3.5129	15:0/18:0; 17:0/16:0
PE (34:1)	718.5384	718.5387	−0.3925	18:1/16:0; 16:1/18:0
PE (34:0)	720.5520	720.5543	−3.2350	17:0/17:0; 15:0/19:0; 16:0/18:0

The iso and anteiso C15 and C17 are the most abundant fatty acids of *B. licheniformis* I89. It is not possible to distinguish the linear C15 and C17 from their branched isomers by LC-MS and MS/MS analysis.

3. Results

The polar lipid profile of *B. licheniformis* I89 was characterized at the molecular level by high-resolution HILIC-ESI-MS, mass accuracy measurements and MS/MS in positive and negative ion modes. The HILIC allowed the separation of several lipid classes (Fig. 1) including PL, GL and phosphoglyceroglycolipids (PGL) (Fig. 2). The lipid species were identified by exact mass measurement and their structural features, as polar head composition and length of the fatty acyl chains, were confirmed by MS/MS spectra interpretation.

3.1. Phospholipid profile

Several classes of PL were identified in *B. licheniformis* I89: PG, lys-PG, CL and PE (Table 1).

PG were identified in negative ion mode, as $[M - H]^-$ ions (Fig. 3a), and in positive ion mode, as $[M + NH_4]^+$ ions (Fig. S1a). The most abundant ions seen in the MS spectra of PG, in negative mode (Fig. 3a), were found at m/z 721.5, 693.5, and 707.5, corresponding to PG (32:0), PG (30:0), and PG (31:0). The MS/MS spectra of the $[M - H]^-$ ions (Fig. 3b) showed the typical product ion at m/z 171.0, assigned as ionized glycerol phosphate polar head, that confirms the presence of a PG molecular species. The MS/MS spectra, in negative mode, provided information about the fatty acyl composition by the identification of the carboxylate anions ($RCOO^-$). The MS/MS spectrum of PG (15:0/17:0) shows the product ions at m/z 241.2 and 269.2 corresponding to the carboxylate anions of the C15:0 and C17:0 FA, respectively (Fig. 3b). These FA were confirmed, as well, by the presence of low abundant product ions at m/z 497.3, arising from loss of C15:0 as ketene ($-R = C=O$) [16]. The iso and anteiso C15 and C17 are characteristic FA of *Bacillus* spp. [16] and were previously found in the FA profile of these samples analyzed by GC-MS [32]. However, it is not possible to distinguish the linear C15 and C17 from their branched isomers by LC-MS and MS/MS analysis. PG were also identified in the LC-MS/MS data uof the $[M + NH_4]^+$ ions (Fig. S1a). The MS/MS of PG (32:0) assigned as PG (15:0/17:0) at m/z 740.5 is given as an example (Fig. S1b), showing the combined neutral loss of NH_3 (-17 Da) and the glycerol phosphate polar head ($17 + 172$ Da), with the formation of the product ion at m/z 551.5.

Lys-PG were identified in both negative and positive ion modes, as $[M - H]^-$ and $[M + H]^+$ ions, respectively. In the LC-MS spectra, in negative ion mode, the most abundant species were found at m/z 849.6, 821.6, and 835.6, corresponding to lys-PG (32:0), lys-PG (30:0), lys-PG (31:0), respectively (Fig. 3c). The analysis of the MS/MS spectra of the $[M - H]^-$ ions (Fig. 3d) showed the characteristic product ion at m/z 145.1, assigned to the deprotonated lysine. The fatty acyl composition was confirmed by the $[M - H]^-$ ions that showed the typical carboxylate anions ($RCOO^-$). The MS/MS spectrum of lys-PG (32:0), assigned as lys-PG (15:0/17:0) (Fig. 3d) showed the product ions at m/z 241.2 and 269.2 assigned as the $RCOO^-$ ions of the FA C15:0 and C17:0, respectively. Lys-PG were also confirmed by MS/MS of the $[M + H]^+$ ions (example of lys-PG (32:0) in Fig. S1d) that showed the typical neutral loss of 300 Da, formed by loss of the polar head group. In this spectrum, it is seen the product ion at m/z 301.1, typical of lys-PG class,

that corresponds to the protonated lysyl-glycerolphosphate head group.

CL were identified in the LC-MS spectra in negative ion mode as mono-charged ions ($[M - H]^-$, Fig. 3e) and double-charged ions ($[M - 2H]^{2-}$, Fig. S1g). Several molecular species of CL were identified as $[M - H]^-$ ions (Fig. 3e), being the most abundant found at m/z 1323.9, 1351.9, 1337.9 and 1309.9, corresponding to CL (62:0), CL (64:0), CL (63:0) and CL (61:0), respectively. The typical fragmentation of CL, as $[M - H]^-$ ions (Fig. 3f), is presented for the MS/MS spectrum of the deprotonated molecule at m/z 1323.9, which corresponds to CL (15:0/17:0/15:0/15:0). This spectrum showed the ions at m/z 619.4 and 647.5 that correspond to the anions of the PA fragments, $[PA-(30:0)-H]^-$ and $[PA-(32:0)-H]^-$, respectively (Fig. 3f). The fatty acyl composition was confirmed by the identification of the $RCOO^-$ product ions at m/z 241.2 and 269.2 corresponding to the FA C15:0 and C17:0, respectively. The MS/MS spectrum of the $[M - 2H]^{2-}$ ions (Fig. S1h) of the same CL, at m/z 661.5 showed ions at m/z 241.2 and 269.2, corresponding to the $RCOO^-$ ions mentioned above.

PE molecular species were identified both in positive and negative ion modes, as $[M + H]^+$ and $[M - H]^-$ ions, respectively (Fig. 3g and Fig. S1e). Several molecular species of PE were identified in positive mode (Fig. 3g) and the most abundant ones were found at m/z 692.5, 678.5 and 664.5, corresponding to PE (32:0), PE (31:0), and PE (30:0), respectively. They were confirmed by the analysis of the MS/MS spectra of the $[M + H]^+$ ions by the identification of the typical neutral loss of 141 Da. The MS/MS spectrum of PE (15:0/17:0) (Fig. 3h) showed a product ion at m/z 551.5, formed by the loss of the phosphatidylethanolamine head group (-141 Da) from the precursor $[M + H]^+$ ion at m/z 692.3. The fatty acyl composition was confirmed by the analysis of the MS/MS spectra of the $[M - H]^-$ ions that showed the typical carboxylate anions ($RCOO^-$). Fig. S1f shows the $RCOO^-$ at m/z 241.2 and 269.2, that correspond to the FA C15:0 and C17:0, respectively.

3.2. Glycolipid profile

Two glycolipid classes were assigned in the lipidome of *B. licheniformis* I89: MGDG and DGDG (Table 2).

MGDG were identified as $[M + NH_4]^+$ (Fig. 4a). The most abundant MGDG species were found at m/z 748.6, 734.6 and 720.6, assigned to MGDG (32:0), MGDG (31:0), and MGDG (30:0), respectively. These lipids were confirmed by MS/MS analysis in positive ion mode (Fig. 4b) by the identification of the typical neutral loss of 197 Da, corresponding to the combined loss of a hexose (-180 Da) and the loss of NH_3 (-17 Da). Fig. 4b shows, as an example of the fragmentation of this class of GL, the MS/MS spectrum of MGDG (15:0/17:0), at m/z 748.6, that showed a product ion at m/z 551.5 formed by the loss of hexose combined with the loss of NH_3 . The fatty acyl composition was confirmed by the presence of product ions at m/z 299.3, corresponding to the ion C15:0 (242 Da) plus the glycerol moiety (57 Da), and the ion at m/z 327.3, corresponding to the C17:0 plus 57 Da, also designated $[RCO + 74]^+$ ions [33].

DGDG were identified (Fig. 4c). The most abundant DGDG molecular species were seen in the LC-MS data at m/z 910.6, 896.6 and 882.6 that corresponded to DGDG (32:0), DGDG (31:0) and DGDG (30:0), respectively. They were confirmed by MS/MS analysis by the

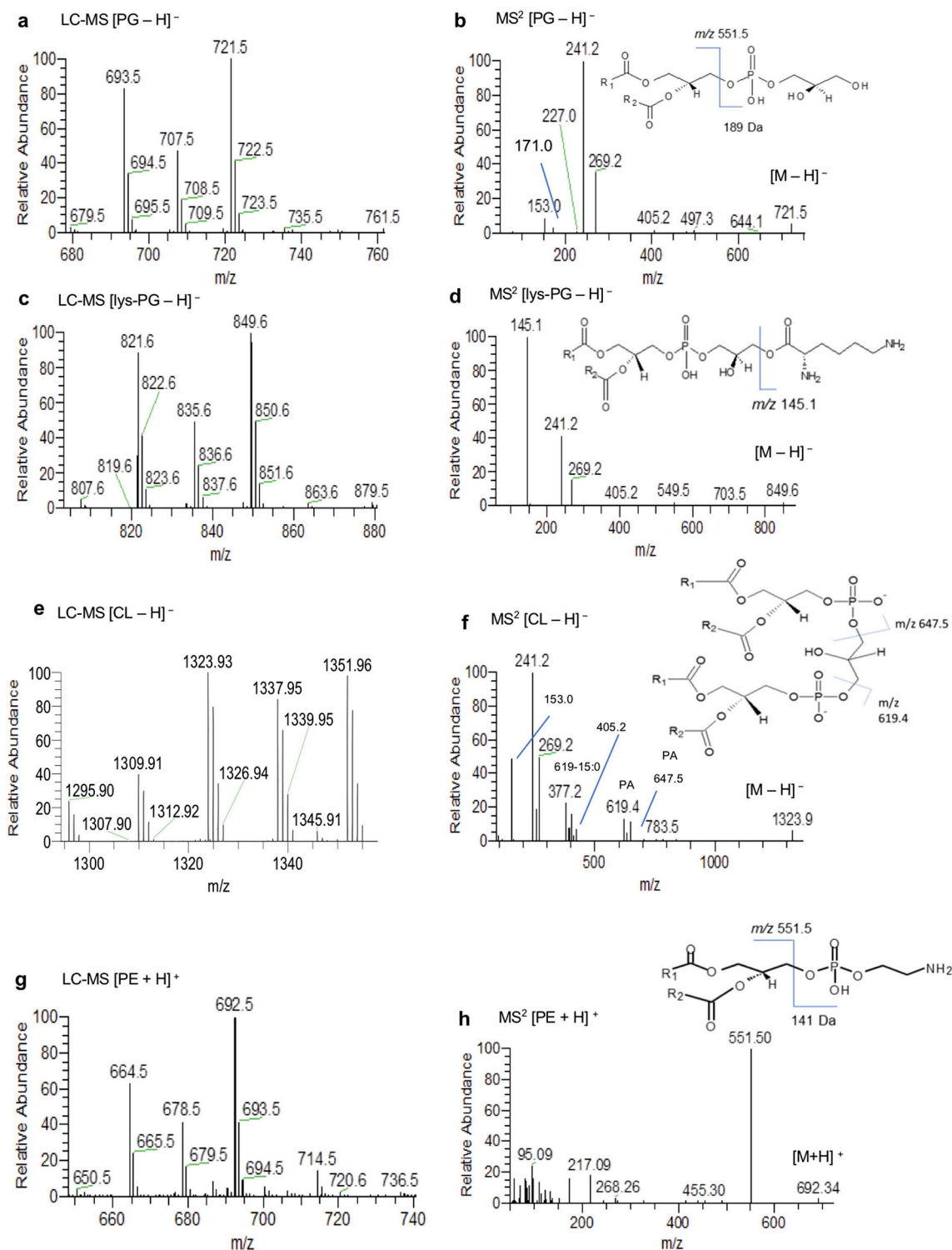


Fig. 3. LC-MS spectra of the phospholipid classes identified in *B. licheniformis* I89 lipidome in the negative ion mode: PG (a), lys-PG (c), CL (e) and in positive ion mode for PE (g). LC-MS/MS spectra and fragmentation pattern of one of the possible isomers of the [M – H][–] ions of PG (17:0/15:0) at *m/z* 721.5 (b), lys-PG (17:0/15:0) at *m/z* 849.6 (d), CL (15:0/15:0/15:0/17:0) at *m/z* 1323.9 (f) and of the [M + H]⁺ ion of PE (17:0/15:0) at *m/z* 692.3 (h).

identification of the typical neutral loss of 359 Da arising from the loss of two hexoses (loss of 162 + 180 Da) combined with the loss of NH₃ (–17 Da). This typical fragmentation pathway can be observed in Fig. 4d, showing the MS/MS spectrum of DGDG (17:0/15:0) at *m/z* 910.6, that led to the formation of the product ion at *m/z* 551.5. The fatty acyl composition was confirmed by the presence of the acylium ions plus 74 ([RCO + 74]⁺), as described for the MGDG class. In the

case of DGDG (17:0/15:0) (Fig. 4d), these product ions can be seen at *m/z* 299.3 that corresponds to [RCO + 74]⁺ of C15:0, and at *m/z* 327.3 that corresponds to [RCO + 74]⁺ of C17:0.

Neutral glycolipids MGDG and DGDG were also detected in LC-MS in negative ion mode as [M + CH₃COO][–] ions (Figs. S2a and S2c). They were confirmed by mass accuracy (Table 2) and by MS/MS analysis that showed only the carboxylate anions RCOO[–], and not the loss

Table 2

Molecular species of glycolipids from *B. licheniformis* I89 identified by LC-MS in positive and negative ion modes.

Mass spectrometry data				
Lipid Group	Observed m/z value	Calculated m/z value	Mass deviation [ppm]	Fatty acid chain
Diglycosyldiacylglycerols $[M + NH_4]^+$				
DGDG (29:0)	868.5975	868.5997	−2.5892	14:0/15:0
DGDG (30:0)	882.6130	882.6154	−2.7181	15:0/15:0; 14:0/16:0
DGDG (31:1)	894.6130	894.6154	−2.6816	15:0/16:1
DGDG (31:0)	896.6283	896.6310	−3.0659	15:0/16:0; 17:0/14:0
DGDG (32:1)	908.6270	908.6310	−4.4562	15:0/17:1
DGDG (32:0)	910.6430	910.6467	−4.0619	15:0/17:0
DGDG (33:0)	924.6590	924.6623	−3.6219	15:0/18:0; 17:0/16:0; 19:0/14:0
DGDG (34:0)	938.6750	938.6780	−3.1949	17:0/17:0; 19:0/15:0
Diglycosyldiacylglycerols $[M + CH_3COO]^-$				
DGDG (29:0)	909.5792	909.5787	0.5717	14:0/15:0
DGDG (30:0)	923.5946	923.5943	0.2923	15:0/15:0; 14:0/16:0
DGDG (31:0)	937.6101	937.6100	0.1280	15:0/16:0; 14:0/17:0
DGDG (32:0)	951.6251	951.6256	−0.5569	15:0/17:0
DGDG (33:0)	965.6408	965.6413	−0.4971	16:0/17:0
Monoglycosyldiacylglycerols $[M + NH_4]^+$				
MGDG (29:0)	706.5442	706.5469	−3.8554	14:0/15:0
MGDG (30:0)	720.5600	720.5620	−2.7756	15:0/15:0
MGDG (31:0)	734.5754	734.5777	−3.1311	15:0/16:0
MGDG (32:0)	748.5917	748.5933	−2.1373	15:0/17:0
MGDG (33:0)	762.6069	762.6095	−3.4408	16:0/17:0
MGDG (34:0)	776.6214	776.6252	−4.8595	17:0/17:0
Monoglycosyldiacylglycerols $[M + CH_3COO]^-$				
MGDG (29:0)	747.5257	747.5259	−0.2074	14:0/15:0
MGDG (30:0)	761.5408	761.5415	−0.9258	15:0/15:0
MGDG (31:0)	775.5568	775.5572	−0.4577	15:0/16:0
MGDG (32:0)	789.5723	789.5728	−0.6396	15:0/17:0
MGDG (33:0)	803.5880	803.5885	−0.5662	16:0/17:0

The iso and anteiso C15 and C17 are the most abundant fatty acids of *B. licheniformis* I89. It is not possible to distinguish the linear C15 and C17 from their branched isomers by LC-MS and MS/MS.

of the sugar moieties (Figs. S2b and S2d).

3.3. Phosphoglyceroglycolipid profile

PGL, well known glycolipid anchors of lipoteichoic acid [34], were identified in *B. licheniformis* I89 as mono-alanylated lipoteichoic acid primer (LTAP-Ala) and lipoteichoic acid primer (LTAP) (Table 3).

LTAP were identified in negative ion mode as $[M - H]^-$ ions (Fig. 5a). The most abundant molecular species were found at m/z 1045.6, 1031.6 and 1017.6, assigned as LTAP (32:0), LTAP (31:0), LTAP (30:0), respectively. The MS/MS spectra of this class (Fig. 5b) showed the typical product ions: at m/z 79.0, that corresponds to the deprotonated ion of the phosphate residue; at m/z 153.0, that corresponds to the glycerolphosphate residue; and at m/z 171.0, that corresponds to glycerolphosphate. These product ions can be observed in the MS/MS spectrum of the $[M - H]^-$ ion of LTAP (15:0/15:0) at m/z 1017.6 (Fig. 5b). FA were identified by the presence of the $RCOO^-$ ions at m/z 241.2 corresponding to C15:0.

LTAP-Ala were identified in negative ion mode, as $[M - H]^-$ ions

(Fig. 5c) and confirmed by MS/MS analysis (Fig. 5d). The molecular species identified were found at m/z 1116.6, 1088.6 and 1102.6, assigned as LTAP-Ala (32:0), LTAP-Ala (30:0), LTAP-Ala (31:0), respectively. This class was identified by the typical neutral loss of alanine (−89 Da), and the product ion at m/z 88.0 that corresponds to the anion of the terminal ester linked alanine [34]. The product ions at m/z 79.0 and at m/z 153.0 were also observed and confirmed the presence of phosphate and glycerolphosphate moieties, respectively. The FA composition was confirmed by the identification of the $RCOO^-$ ions at m/z 241.2 and 269.2 that matched with the expected LTA-Ala (15:0/17:0) composition.

3.4. Lipid profile is growth phase-dependent

The lipid profile was analyzed in the three growth phases: lag, exponential and stationary, at 37 °C. The same lipid classes and molecular species were identified in all growth phases, but there were changes in the relative abundances of the lipid species in all PL classes. Significant differences in PG molecular species were observed for PG (30:0) and PG (32:0) ($p < 0.001$, ANOVA) and PG (31:0) ($p < 0.05$, ANOVA) (Fig. 6a). PG (30:0) significantly increased along the growth phases while PG (32:0) decreased from the lag to the exponential and stationary phases. PG (31:0) decreased in stationary phase (Fig. 6a). As for PG, significant differences in the relative abundance of lys-PG (32:0) and lys-PG (30:0) were observed ($p < 0.001$, ANOVA) (Fig. 6b). Lys-PG (30:0) increased over the growth phases, while lys-PG (32:0) decreased from the lag to the stationary phase. Significant differences in CL molecular species were observed for CL (64:0) (Fig. 6c). The CL (64:0) decreased from the lag to the exponential phase and increased from the exponential to the stationary phase. The differences in the relative abundance of PE occurred in PE (30:0) and PE (32:0) (Fig. 6d). PE (30:0) increased along the growth phases, while PE (32:0) decreased throughout the growth phases.

For the GL, significant differences in the relative abundance of MGDG were observed in MGDG (30:0) which increased from the lag phase to the stationary phase (Fig. 6e). Significant differences were observed in the relative abundance of DGDG (30:0) and DGDG (32:0) (Fig. 6f). DGDG (30:0) increased throughout the growth phases, while DGDG (32:0) decreased (Fig. 6f).

Regarding PGL classes, the differences in the relative abundances occurred in LTAP (32:0) (Fig. 6g), that decreased from the lag to the exponential phase and increased from the exponential to the stationary phase. The differences in LTAP-Ala class occurred in LTAP-Ala (30:0) ($p < 0.001$, ANOVA) and LTAP-Ala (32:0) ($p < 0.05$, ANOVA) (Fig. 6h). LTAP-Ala (30:0) increased throughout the growth phases and LTAP-Ala (32:0) decreased throughout the phases.

4. Discussion

Membrane lipid homeostasis and adaptation to changing environmental conditions are essential for bacterial survival [1]. Lipid metabolism and lipid profile can change depending on growth conditions, such as temperature [35]. Modifications in the lipid composition of bacterial membrane were associated with changes in the profile of FA or the ratio of *iso* to *anteiso* chains [35]. Also, it is known that changes in the lipid composition, not only at the FA level but also in the PL and GL profiles, can affect membrane properties, bacterial survival and pathogenicity [36]. However, the lipidome of most bacteria is still unknown [1].

The polar lipidome of *B. licheniformis* I89 was studied herein. Polar lipids were extracted and analyzed, both in negative and positive ion modes, by means of an LC-MS-based lipidomic platform. This approach allowed the identification of several classes of polar lipids providing a more complete lipidomic signature of this bacterial strain. Previous studies performed in *Bacillus* spp. could identify only three PL classes (PG, PE and lys-PG) and one GL class (DGDG) [19]. In this study, four

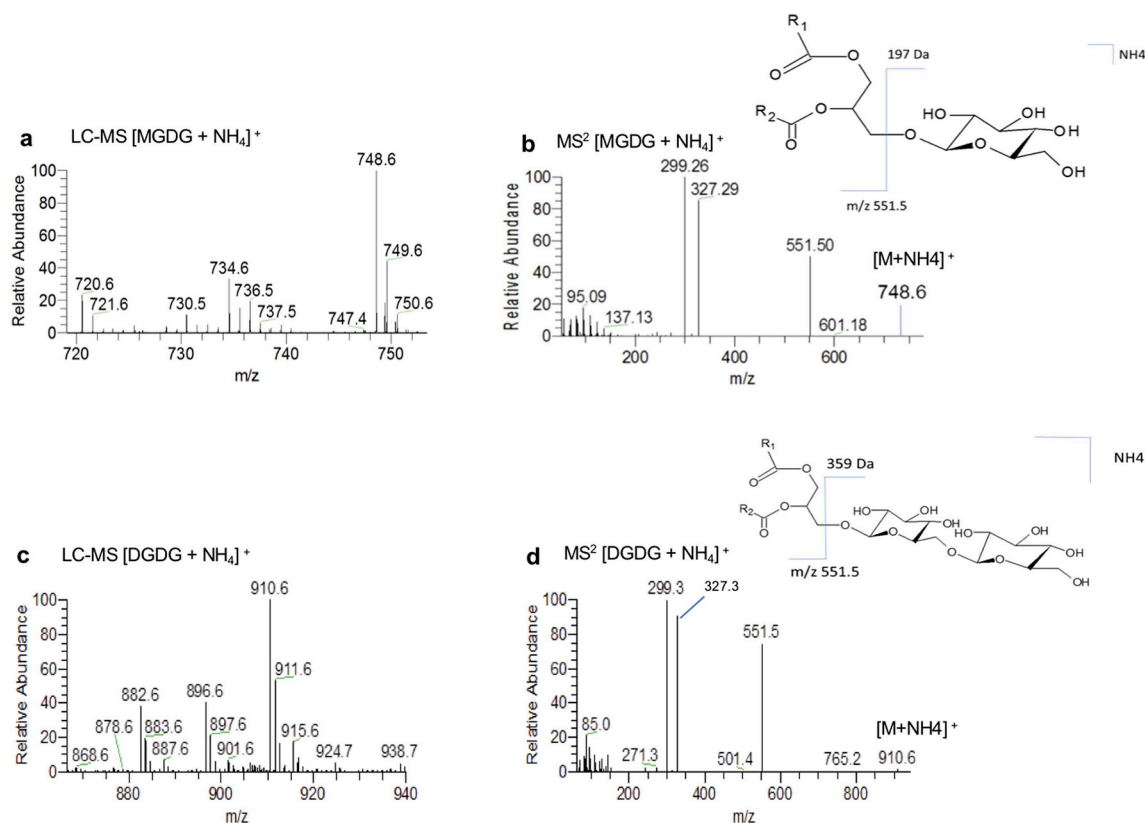


Fig. 4. LC-MS spectra of glycolipids identified in *B. licheniformis* I89 lipidome: MGDG (a) and DGDG (c). LC-MS/MS spectra acquired in positive ion mode and fragmentation pattern of one of the possible isomers of MGDG (17:0/15:0) at m/z 748.6 (b) and DGDG (17:0/15:0) at m/z 910.6 (d).

Table 3

Molecular species of phosphoglyceroglycolipids from *B. licheniformis* I89 identified by LC-MS in negative ion mode.

Mass spectrometry data				
Lipid Group	Observed m/z value	Calculated m/z value	Mass deviation [ppm]	Fatty acid chain
Diglycosyldiacylglycerols - Phospho- Glycerol $[M - H]^-$				
LTAP (29:0)	1003.5607	1003.5607	0.0369	14:0/15:0
LTAP (30:0)	1017.5760	1017.5763	-0.3076	15:0/15:0
LTAP (31:0)	1031.5915	1031.5920	-0.4488	15:0/16:0
LTAP (32:0)	1045.6083	1045.6076	0.6570	15:0/17:0
Diglycosyldiacylglycerols - Phospho- Glycerol - Alanyl $[M - H]^-$				
LTAP-Ala (30:0)	1088.6089	1088.6134	-4.1585	15:0/15:0
LTAP-Ala (31:0)	1102.6263	1102.6291	-2.5185	15:0/16:0
LTAP-Ala (32:0)	1116.6445	1116.6447	-0.2033	15:0/17:0

The iso and anteiso C15 and C17 are the most abundant fatty acids of *B. licheniformis* I89. It is not possible to distinguish the linear C15 and C17 from their branched isomers by LC-MS and MS/MS analysis.

PL classes, two GL classes, and two PGL classes were identified. The PL classes identified in *B. licheniformis* I89 were PG, PE, lys-PG, and CL. These lipid classes were already reported in other studies of *Bacillus* spp. and also of other Gram-positive bacteria. However, CL and PE classes have never been reported in the lipidome of *B. licheniformis*, contrarily to PG and lys-PG [23]. CL is a universal component of energy generating membranes, it plays an important function in diverse physiological processes, including stability and localization of proteins and

protein complexes, formation of membrane microdomains and the production of membrane potential [37]. Besides, CL exhibits a cone-shaped architecture that locates at regions of negative membrane curvature [38,39] responsible for modulating membrane properties and function, and protein location in the cellular membrane.

Lys-PG are aminoacylated PG commonly present in bacterial cytoplasmic membranes and have a key role in the stabilization of the membranes [40]. In *S. aureus*, they seem to play a role in the resistance to cationic antimicrobial peptides and to the lipopeptide antibiotic daptomycin. This effect seems to be related with the decreased susceptibility of the membrane to these compounds due to the partial neutralization of the cellular membrane by the cationic headgroup of lys-PG [41]. These membrane lipids also provide protection against bacitracin, aminoglycosides, and some β -lactams [42].

DGDG were already identified in members of the *Bacillus* spp. and other Gram-positive bacteria [19,43,44]. MGDG were reported in other Gram-positive bacteria [17] but not in the lipidome of *B. licheniformis* or another *Bacillus* spp.

Lipoteichoic acid (LTA) and alanyl-lipoteichoic acid (Ala-LTA) classes, identified in this study, were already reported in Gram-positive bacteria, including *Bacillus* spp. Aminoacylated lipids were shown to play a role in surface charge modulation of Gram-positive bacteria [45]. LTA are recognized as immunomodulating effector molecules and can induce an *in vitro* pro-inflammatory response in immune cells [46]. This response occurs due to D-alanyl substitution of the LTA backbone, its glycolipid anchor [47,48]. Thus, the absence of functional LTA in the bacterial membrane improves the bacterial anti-inflammatory ability [46,49].

The polar lipid profile described in this study for *B. licheniformis* I89 agrees with data reported previously for Gram-positive bacteria and for some *Bacillus* species, but those works reported only few groups of lipids in the bacteria [16,19,50]. Gram-positive bacteria are known to contain PL bearing amino acids in the head group, such as lysine,

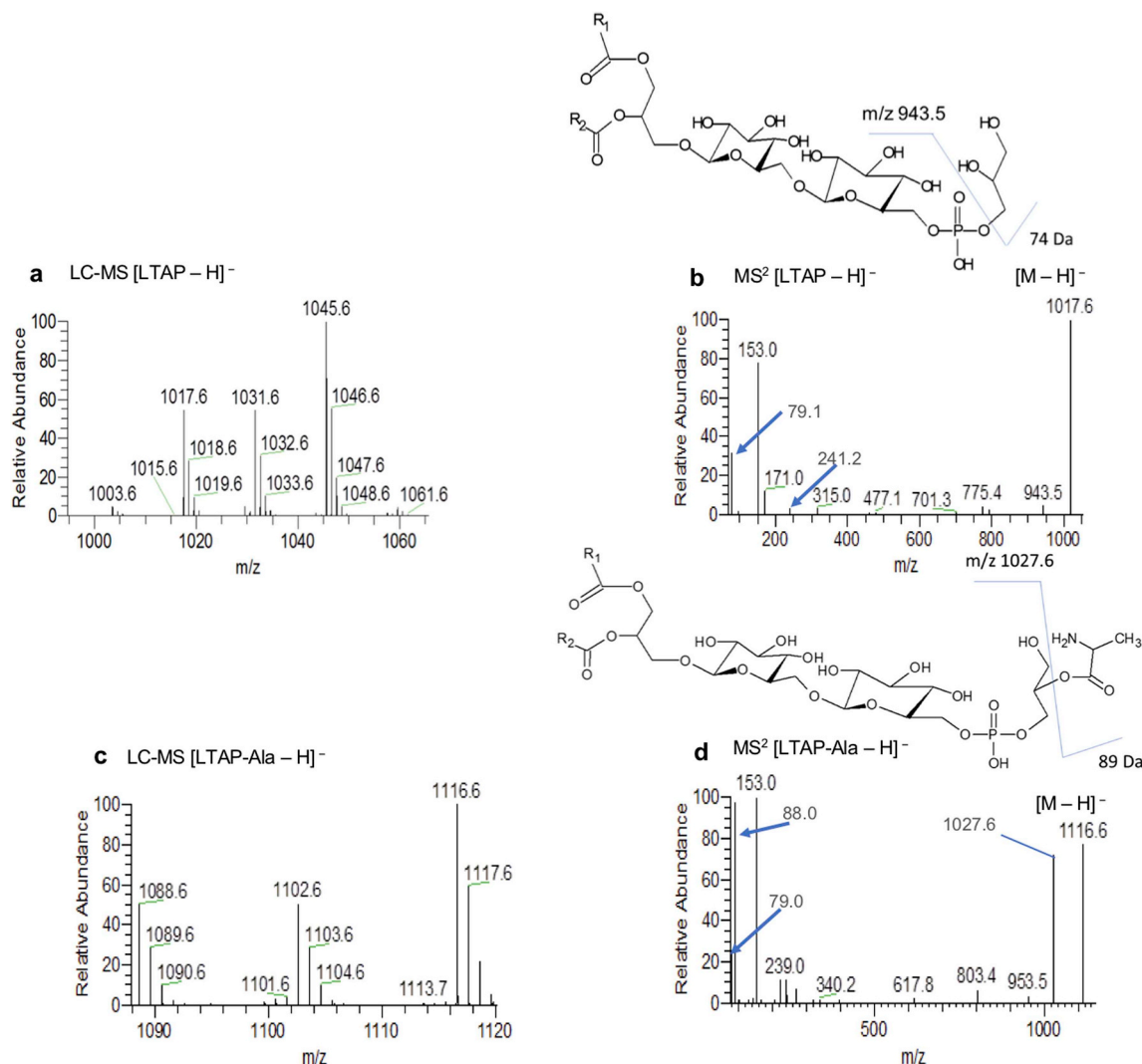


Fig. 5. LC-MS spectra of phosphoglyceroglycolipids identified in *B. licheniformis* I89: LTAP (a) and LTAP-Ala (c). LC-MS/MS spectra acquired in negative ion mode and fragmentation pattern of one of the possible isomers of the $[M - H]^-$ ions of LTAP (15:0/15:0) at m/z 1017.6 (b) and LTAP-Ala (17:0/15:0) at m/z 1116.6 (d).

alanine and ornithine [51]. DGDG and MGDG were reported for other *Bacillus* spp., identified by thin-layer chromatography or by direct MS analysis [18,23].

The identification of the polar lipidome of bacteria is important to provide information about their adaptation mechanisms, namely to developing antibiotic resistance. Previous studies reported a correlation between lipid composition and antibiotic resistance in bacteria [50,52]. In *Enterococcus faecalis*, a Gram-positive, opportunistic, pathogenic bacterium [50], antibiotic resistance was correlated with a decrease in PG and lys-PG levels which, most probably, provide resistance to cationic antimicrobial peptides [50]. Changes in PL profile were also observed in MG1655, DPB635 and DPB636 *E. coli* strains, after exposure to the antibiotic norfloxacin, with up regulation of FA and down regulation of glycerophospholipids [52]. Other works reported that the susceptibility to different antibiotics in the pathogenic bacteria *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* depends on the variations of the lipidome induced by nutrition depletion [53].

5. Conclusions

The profiling of the polar lipidome of *Bacillus* species and other Gram-positive bacteria by LC-MS is still in its infancy. In the lipidome of *B. licheniformis* I89, one hundred and fourteen molecular species of

polar lipids were identified and structurally characterized by LC-MS, comprising phospholipids (PG, lys-PG, PE, and CL), glycolipids (MGDG, DGDG), and phosphoglyceroglycolipids (LTAP, LTA-Ala). Membrane lipid composition of this strain is significantly modified during the different growth phases. Thus, the results in the present work, obtained through LC-MS with high resolution MS, are promising to understand the adaptation of the lipid metabolism of *Bacillus*, under different growth conditions, that can be useful to understand mechanisms of resistance and also for taxonomy classification.

Conflicts of interest

None.

Acknowledgment

Thanks are due to University of Aveiro, Fundação para a Ciência e a Tecnologia (FCT)/MEC, European Union, QRN, COMPETE for the financial support to the Organic Chemistry, Natural and Agrofood Products (QOPNA) research Unit (FCT UID/UI/00062/2013), Portuguese Mass Spectrometry Network, RNEM (LISBOA-01-0145-FEDER-402-022125) and Centre for Environmental and Marine Studies (CESAM) (UID/AMB/50017/2013), through national funds and, where

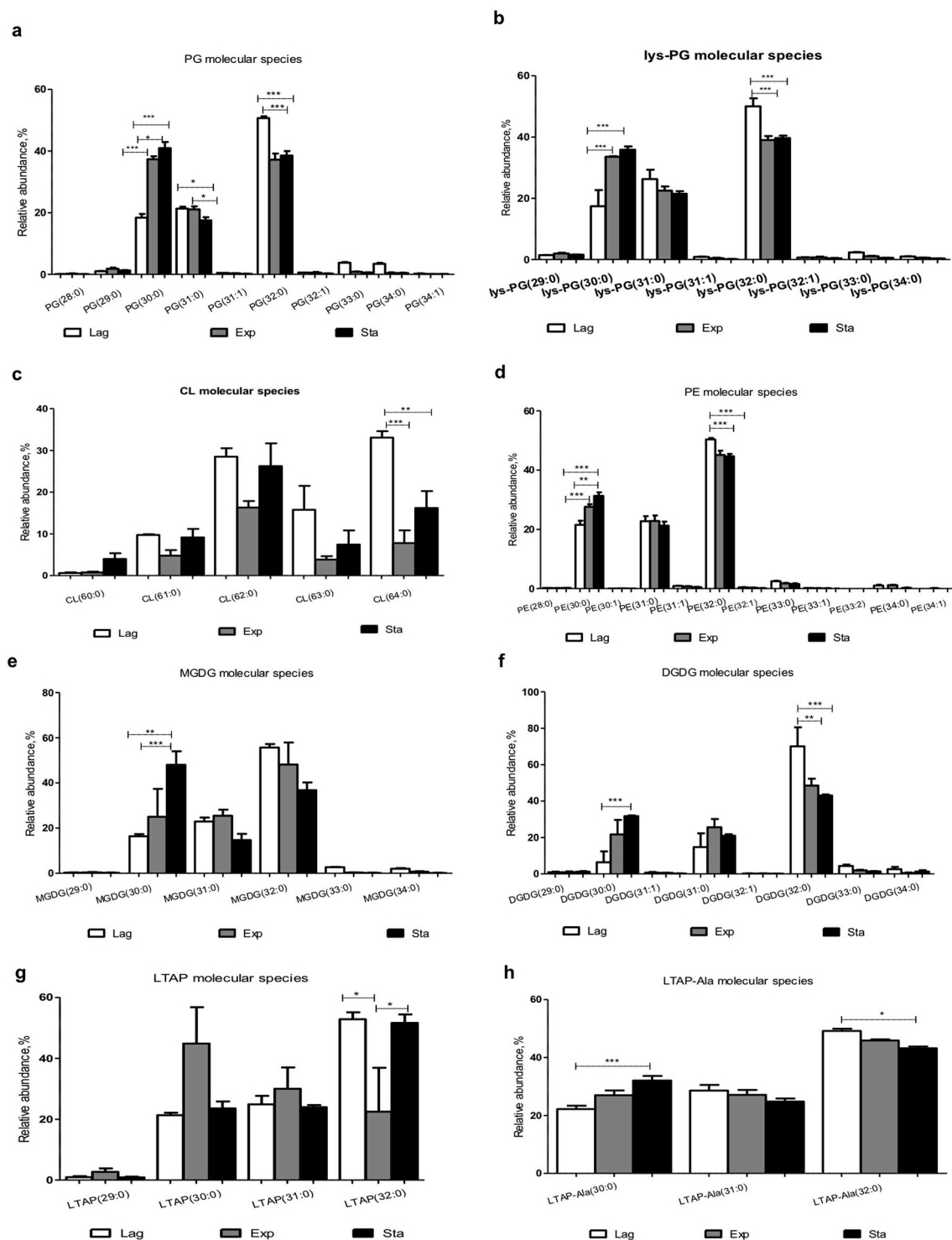


Fig. 6. Comparison of the polar lipid species of *B. licheniformis* 189 between the lag, exponential (Exp) and stationary (Sta) growth phases at 37 °C: (a) PG, (b) lys-PG, (c) CL, (d) PE, (e) DGDG, (f) MGDG, (g) LTAP and (h) LTAP-Ala. Values are means \pm standard deviation, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (ANOVA).

applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement, and also to the Portuguese Mass Spectrometry Network (REDE/1504/REM/2005). Celestina Lopes is grateful to Calouste Gulbenkian Foundation for her PhD grant (process number 135624). Joana Barbosa (SFRH/BD/97099/2013), Elisabete Maciel (SFRH/BPD/104165/2014), Elisabete da Costa (SFRH/BD/52499/2014) and Eliana Alves (SFRH/BPD/109323/2015) are grateful to FCT for their grants.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.abb.2018.12.024>.

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CHAPTER IV

IV. CHANGES IN *Bacillus licheniformis* 189 MEMBRANE LIPID COMPOSITION AFTER EXPOSURE TO VANCOMYCIN

IV. Introduction

B. licheniformis I89 was isolated from an hot spring environment in São Miguel, Azores, Portugal. It is a non-pathogenic Gram-positive, endospore-forming bacterium. of the genus *Bacillus* that belongs to the *B. subtilis* group. Members of this genera produce valuable compounds, such as proteases, amylases, surfactants, immunosuppressors, antimicrobials (e.g., lichenicidin, bacitracin, surfactin), lipids, among others. *B. licheniformis* I89 produces lichenicidin, a lantibiotic which exhibits activity against clinically relevant *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant enterococcus strains [1]. Vancomycin is a glycopeptide antibiotic that is used as a last resort in the treatment of serious infections caused by Gram-positive pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), enterococci, and *Clostridium difficile* [2]. It binds to the C-terminal D-Ala-D-Ala residues present in the pentapeptide of lipid II-linked disaccharides, to the phosphatidylglycerol (PG) precursors, and to the non-crosslinked peptides of the nascent peptidoglycan [3]. Vancomycin inhibit the synthesis of peptidoglycan precursors of the bacterial cell wall by blocking the transglycosylation step, subsequently affecting transpeptidation [4,5] and thus interfering with cell membrane function[6]. Both the transglycosylation and transpeptidation steps are essential for bacterial cell wall cross-linking. Vancomycin binds to the bacterial cell membrane through direct interaction with the phospholipid head group and this association depends very much on the specific type of lipids and the curvature of the membrane [6]. Yet, other compounds exist which have activity against Gram-positive bacteria and they usually act at the level of inhibition of the cell wall synthesis.

In the presence of antibiotic concentrations lower than the minimum inhibitory concentration (MIC) cells survive, but we suspect that this might affect the lipid profile of the bacterium's cell wall and that was precisely what we have investigated. To that end, we subjected *B. licheniformis* I89 cells, to sub-inhibitory (lower than the MIC) concentrations of vancomycin, which were previously determined, and then we have evaluated the change of the lipid profile of those cells. Variation in the lipidome following exposure to vancomycin was determined through the analysis of phospholipids and fatty acids profiles by LC-MS and GC-

MS, respectively. Three conditions were studied: control and two different vancomycin concentrations. The effect of vancomycin was also evaluated in two different growth phases, lag and exponential.

IV. 2. Materials and Methods

IV.2.1 Determination of minimum inhibitory concentration for vancomycin (MIC)

Vancomycin (Alfa Aesar) was dissolved in ultrapure water and sterilized by filtration using cellulose acetate membrane filters. A stock solution of vancomycin was prepared (10 mg/ml) and serial dilutions, ranging from 0.016 to 16.0 µg/mL, were prepared in Mueller-Hinton broth (Oxoid) medium. The bacterial suspensions were prepared as follows: *B. licheniformis* I89 and *S. aureus* ATCC 29213 strains were grown in TSB medium at 37°C, 180 rpm, until OD_{625nm} 0.08-0.13. The OD_{625nm} of the cultures was adjusted approximately to 0.1 with TSB medium (equivalent to the 0.5 McFarland standard). To obtain an inoculum with a final concentration equal to 5×10^6 CFU/mL, cultures were diluted 1:100 in TSB medium. Following the recommendations given by ISO (ISO, 2006), this dilution was plated on agar plates to confirm that the bacterial culture was at a final concentration of 5×10^6 CFU/mL. Then, 50 µL of this diluted culture was added to 50 µL of each antibiotic dilution. Bacterial growth was visually checked after 24h of incubation at 37 °C for both strains. For the reference strain *S. aureus* ATCC 29213, vancomycin was used as quality control. The assay was validated based on QC breakpoint tables established by CLSI (2016).

IV.2.2 Growth of *B. licheniformis* I89 in the presence of vancomycin

B. licheniformis I89 cultures were prepared in medium M: 10 g L⁻¹ of NaCl, 10 g L⁻¹ of tryptone, 5 g L⁻¹ of yeast extract, 10 g L⁻¹ of KH₂PO₄, with a final pH of 6.5, adjusted with

NaOH [7]. An overnight pre-culture was prepared as follows: a single colony was inoculated in 10 mL of medium M and the cultures were allowed to grow overnight at 37 °C, 200 rpm until the OD_{600nm} reached approximately 0.9. Then, 1 mL of this pre-culture was used to inoculate 100 mL of fresh medium M, in triplicate. Vancomycin was added to the test conditions at the final concentrations of 0.008 µg/mL or 0.125 µg/mL; no vancomycin was added to the control cultures. Bacterial cells were allowed to grow at 37 °C, 200 rpm, until they reached the lag phase (after 3 - 4 h incubation, OD_{600nm} approx. 0.5) or the exponential phase (after 16 h incubation, OD_{600nm} 1.7 – 2.0). After growth, the cells were harvested at 6500 g for 5 min, at room temperature. The supernatants were discarded, and the cellular pellets were stored at -20 °C until further use. The procedure was done in triplicate for each growth phase at 37 °C.

IV.2.3 Lipid extraction

The bacterial lipids were extracted from the pellets stored at -20 °C, according to Alves and co-workers [8]. Briefly, 6.5 mL of chloroform/methanol (2:1, by volume) were added to the bacterial cells previously suspended in 2 mL of milli-Q water, in glass centrifuge tubes. The mixture was well homogenized by vigorously inverting the tubes several times and incubated on ice for 210 min. The samples were centrifuged at 568 Xg for 10 min (Mixtasel, JP Selecta S.A., Barcelona, Spain) at room temperature to separate the phases: an aqueous upper phase and an organic lower phase from which the lipids were obtained. After transferring the organic phase to a clean tube, the extraction was repeated twice. The extracts were stored in a 2 mL amber glass vial under a nitrogen atmosphere at -20 °C until use.

IV.2.4 Quantification of phospholipids by phosphorus assay

The quantification of phospholipids in the total lipid extracts was done by measuring the amount phosphorus [9]. Briefly, 250 μL of 70% perchloric acid was added to 10 μL of each sample, that was previously dried under a nitrogen stream, in glass tubes. The samples were incubated at 180 $^{\circ}\text{C}$ during 60 min in a heating block (Block Heater SBH200D/3, Stuart®, Bibby Scientific Ltd., Stone, UK), followed by cooling to room temperature. Then, 825 μL of water, 125 μL of 2.5 % aqueous solution of ammonium molybdate, and 125 μL of 10% aqueous solution of ascorbic acid were, then, added to the samples, mixing well after each addition. A standard curve of phosphate was prepared, in parallel, using standards from 0.1 to 2 μg of phosphate (standard solution of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 504 mg L^{-1} of water, i.e., 100 μg of phosphorus mL^{-1}) and underwent the same treatment as the samples. Samples and standards were, afterwards, incubated for 10 min at 100 $^{\circ}\text{C}$ in a water bath (Precistern, JP Selecta S.A., Barcelona, Spain). The absorbance of samples and standards was measured at 797 nm, at room temperature, in a microplate UV-Vis spectrophotometer (Multiskan GO, Thermo Scientific, Hudson, NH, USA).

IV.2.5 Fatty acid analysis by gas chromatography-mass spectrometry (GC-MS)

The total fatty acyl substituents were analyzed after transmethylation of total lipid extracts (30 μg). The FA methyl esters (FAME) were prepared using a methanolic solution of potassium hydroxide (2.0 M), according to the methodology previously described by Aued-Pimentel and co-workers (2004). Volumes of 2.0 μL of the hexane solution containing the FAME were submitted to analysis by gas chromatography-mass spectrometry (GC-MS) on an Agilent Technologies 6890 N Network (Santa Clara, CA) equipped with a DB-FFAP column with 30 m of length, 0.32 mm of internal diameter, and 0.25 μm of film thickness (J&W Scientific, Folsom, CA). The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range

m/z 40-500 in a 1s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 80 °C, standing at this temperature for 3 min, a linear increase to 160 °C at 25 °C min⁻¹, followed by a linear increase to 190 °C at 2 °C min⁻¹. The injector and detector temperatures were 220 °C and 280 °C, respectively. Helium was used as carrier gas at a flow rate of 1.4 mL min⁻¹. The sum of the areas of the peaks assigned as FA in the chromatogram was considered as the total amount of FA. To determine the relative content of each FA, areas of each individual peak were divided by the sum of the area of all the peaks identified allowing to determine the relative content of each FA.

IV.2.6 Hydrophilic interaction liquid chromatography - electrospray ionization - mass spectrometry (HILIC-ESI-MS)

Polar lipids were analyzed by HILIC-ESI-MS on a Thermo Scientific AccelaTM HPLC system with an autosampler online coupled to a Q-Exactive[®] mass spectrometer with Orbitrap[®] technology (Thermo Fisher, Scientific, Bremen, Germany). The solvent system consisted of two mobile phases: mobile phase A was acetonitrile/methanol/water, 50:25:25 per volume, with 1 mM ammonium acetate, and mobile phase B was acetonitrile/methanol, 60:40 per volume, with 1mM ammonium acetate. Initially, 0% of mobile phase A was held isocratically for 8 min, followed by a linear increase to 60% of A within 7 min. maintaining the condition for 15 min, returning to the initial conditions after 10 min. A volume of 5 µL of each sample containing 5 µg of lipid extract and 95 µL of mobile phase B was introduced into the column (Ascentis[®] Si , 15 cm × 1 mm, 3 µm, Sigma-Aldrich) with a flow rate of 40 µL min⁻¹ and at 30 °C. The mass spectrometer was operated simultaneously in positive (electrospray voltage 3.0 kV) and negative (electrospray voltage -2.7 kV) ion modes, with a resolution of 70 000 (FWHM) and automatic gain control (AGC) target of 1 x 10⁶. The capillary temperature was 250 °C and the sheath gas flow was 15 U. In MS/MS experiments, a resolution of 17 500 and AGC target of 1 x 10⁵ were used. The cycles consisted of one full scan mass spectrum and ten data-dependent MS/MS scans and were repeated continuously throughout the experiments with the dynamic exclusion of 60 seconds and intensity threshold

of 1×10^4 . Normalized collision energyTM (CE) ranged between 25, 30 and 35 eV. Data acquisition was carried out using the Xcalibur data system (V3.3, Thermo Fisher Scientific, USA). Three bacterial cultures were analyzed independently for each of the two growth phases. The identification of molecular species of polar lipids was based on the assignment of the molecular ions observed in LC-MS spectra and by the identification of the fragmentation pattern of each class observed in the MS/MS spectrum of each ion [10]. To confirm the identification of molecular species, mass accuracy (Qual Browser) was determined with ≤ 5 ppm.

IV.2.7 Data and Statistical analysis

The raw data were processed using the MZmine software 2.32 [11]. First, the mass list was filtered, followed by peak detection and peak processing. During the processing of the raw data, acquired in full MS mode, only peaks with raw intensity upper than $1e4$ and with a mass tolerance of 5 ppm were considered. Peak assignment and ion identification based on mass accuracy were performed against an in-house database. Data integration was expressed by the changes of the relative abundance of molecular species of all classes. Variation on the lipidome of *B. licheniformis* I89 was measured in triplicate in three different conditions (lag, and exponential phases). Results were expressed as mean \pm SD using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests to compare the growth phases, after checking for assumptions. Significant differences were determined in relative percentages of molecular species per class (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Statistical analysis was performed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

IV.3 Results

3.1 MIC determination and establishment of vancomycin assay conditions

B. licheniformis I89 is sensitive to vancomycin with a minimum inhibitory concentration (MIC) of 0,5 µg/mL. The results were interpreted according to the CLSI breakpoints. The effect of the exposure to sub-inhibitory (0.125 µg/mL and 0.008 µg/mL) concentrations of vancomycin on the lipid composition or lipid metabolism was evaluated in different phases of growth (lag and exponential) of *B. licheniformis* I89 cells growing at 37 °C.

IV.3.2 Adaptation of the FA profile of *B. licheniformis* I89 in the presence of vancomycin

We have studied the variation of FA profile of *B. licheniformis* I89 exposed to the sub-inhibitory concentrations of vancomycin above referred. The fatty acid profile was evaluated using GC-MS analysis of FAMES. Total lipid extracts were prepared from cells that grew in the presence of the two different concentrations of vancomycin: 0.008 µg/mL or 0.125 µg/mL and from control cells (no antibiotic added) and then, the relative content of FA was calculated.

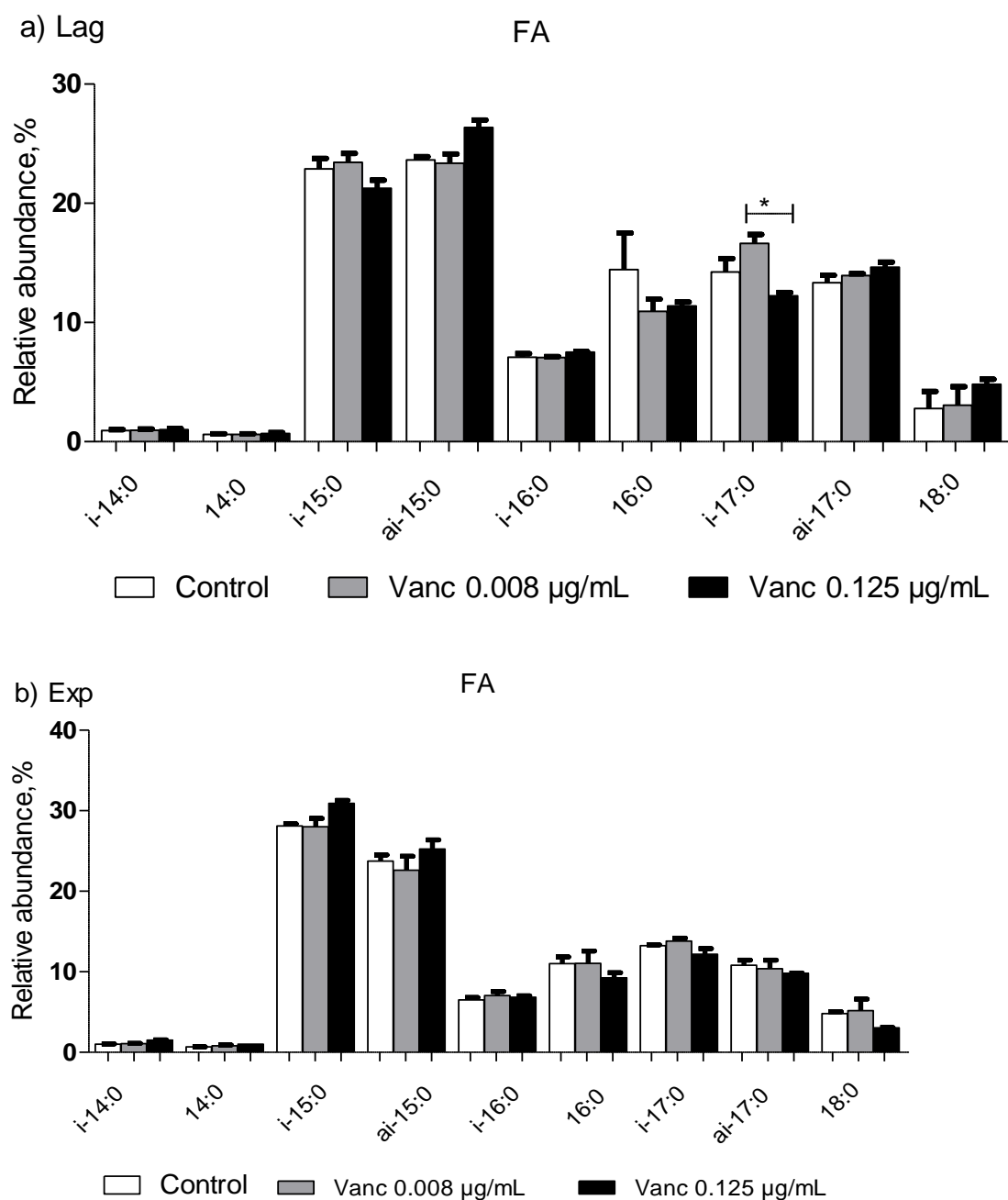


Figure IV.1 - Variation of FA composition of *B. licheniformis* I89 membrane in the lag (a) and exponential (b) phases, after exposure to vancomycin at concentrations of 0.008 and 0.125 µg/mL. Values are means \pm standard deviation, * $p < 0.05$.

The most abundant FA found were i-15:0, ai-15:0, i-17:0, ai-17:0, 16:0 and i-16:0 in the lag and exponential phases, both in the control and in cells that grew in the presence of vancomycin. Significant differences were observed for i-17:0 relative content only in the lag phase (Fig IV.1a), showing a decrease of i-17:0 in the cells that were exposed to the higher concentration of vancomycin. In the exponential phase, no differences were observed in the fatty acid profile (Fig IV.1b).

IV.3.3 Adaptation of polar lipid profile from *B. licheniformis* I89 in the presence of vancomycin

The modern lipidomic approaches, HILIC-ESI-MS, and MS/MS were employed to evaluate the adaptation of the polar lipids of *B. licheniformis* I89 cell membranes, after exposure to vancomycin. With this approach, polar lipids, namely of the molecular species of all the polar lipids classes previously identified in *B. licheniformis* I89 were compared/analyzed. We identified the molecular species of the phosphatidylglycerol (PG), cardiolipins (CL) lipoteichoic acid (LTAP) and mono-alanylated lipoteichoic acid primer (LTAP-Ala), phosphatidylethanolamine (PE), aminoacylated PG (lys-PG), diglycosyldiacylglycerol (DGDG) and monoglycosyldiacylglycerol (MGDG).

The HILIC-ESI-MS mass spectra obtained in the negative ion mode allowed to detect the $[M - H]^-$ ions, in the case of PG, lys-PG, PE, CL, mono-alanylated lipoteichoic acid primer (LTAP-Ala), and lipoteichoic acid primer LTAP. The HILIC-ESI-MS in positive ion mode detected the $[M + NH_4]^+$ ions of the PG, PE, lys-PG, DGDG and MGDG classes. LC-MS/MS was performed for each ion to identify and confirm the respective structures according to the typical fragmentation pattern.

Quantification of each molecular species was performed using MZmine. Molecular species of PG, lys-PG, PE, LTAP-Ala and LTAP were quantified using MS data obtained in negative ion mode, while molecular species of DGDG and MGDG were quantified using MS data obtained in positive mode. Then, quantification data was statistically analyzed. Results gathered in the present work revealed that under the conditions tested, the exposure to

vancomycin induced changes in the amount of some lipid species of polar lipids in the membrane of *B. licheniformis* I89. Comparison of the profile revealed that in the lag phase, significant differences were observed, namely for some lipid species of PG, PE, lys-PG and DGDG classes. The molecular species that showed significant variations were: PG (30:0) ($p < 0.05$), PG (31:0) ($p < 0.01$, $p < 0.05$), PG (33:0) ($p < 0.05$), PG (34:0) ($p < 0.05$) (Fig. IV.2a), lys-PG (30:0) ($p < 0.01$), lys-PG (32:0) ($p < 0.01$) (Fig. IV.2b), PE (30:0) ($p < 0.001$), PE (32:0) ($p < 0.01$) (Fig. IV.2c), and DGDG (32:0) ($p < 0.01$) (Fig. IV.2d). The content of PG (30:0) and PG (31:0) significantly increased after vancomycin exposure. In the case of PG (30:0), there is an increase at higher vancomycin concentration in comparison with lower vancomycin concentration. Likewise, PG (31:0), is present in a higher amount when the *B.licheniformis* I89 cells are grown in the presence of a higher concentration of vancomycin, when compared to the control and with the lowest concentration of vancomycin. PG (33:0) and PG (34:0) significantly increased after vancomycin exposure. PG (33:0) decreased significantly after exposure to higher concentrations of vancomycin when compared to the control and to the lower concentration of vancomycin, while in PG (34:0) there is a decrease in comparison to the lower concentration in the lag phase.

Lys-PG (30:0) and PE (30:0) content significantly increased when the cells are grown at the higher concentration of vancomycin when compared to the control, whereas the opposite is observed for lys-PG (32:0) and PE (32:0) which decreased at the higher concentration of vancomycin.

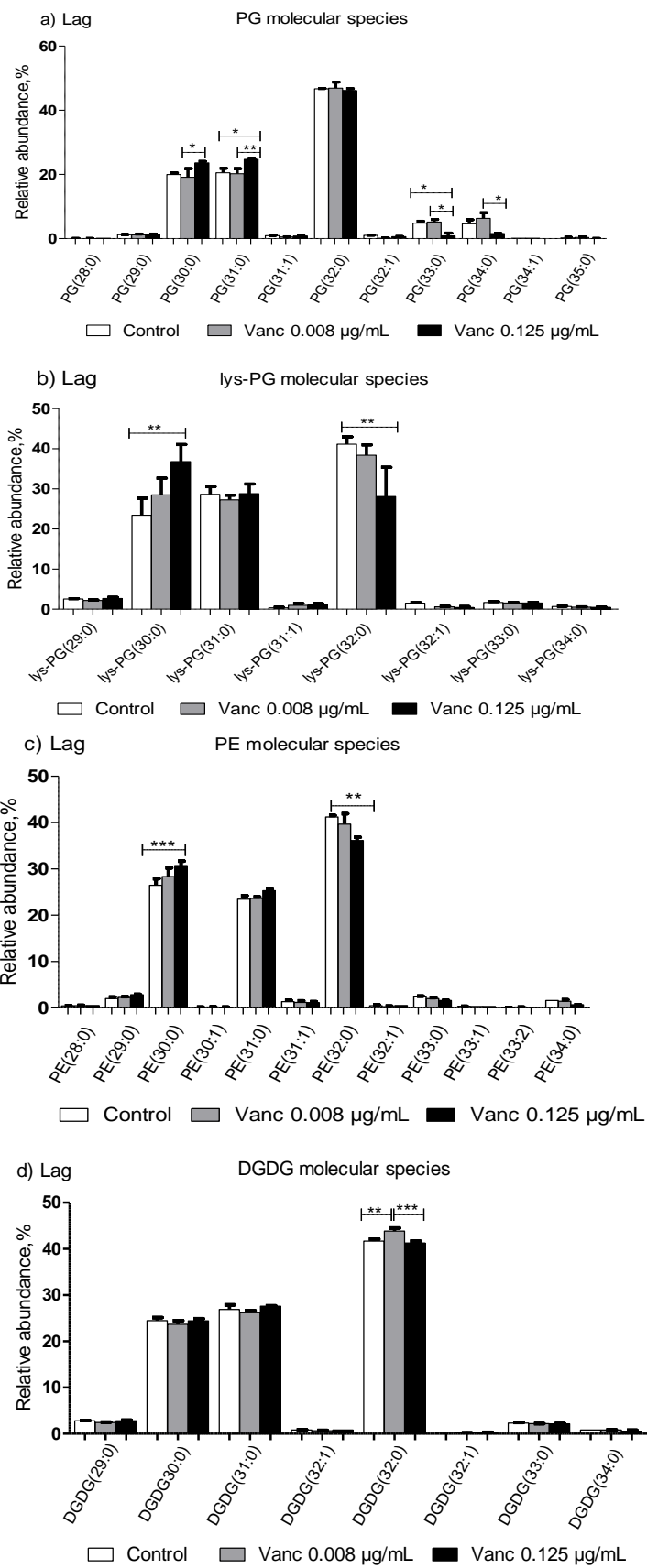


Figure IV. 2 Variation of *B. licheniformis* I89 membrane polar lipid composition in the lag phase, after exposure to vancomycin concentrations of 0.008 and 0.125 $\mu\text{g/mL}$. Variation was observed for the polar lipid classes PG (a), lys-PG (b) PE (c), and DGDG(d). Values are means \pm standard deviation, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The relative content of DGDG (32:0) significantly increased on cells grown at the lower vancomycin concentration in comparison to the control and it decreased in cells grown at the higher vancomycin concentration. As for the exponential growth phase, significant differences were observed only in some molecular species of Lys -PG and PE, namely: lys-PG (30:0) ($p < 0.01$) (Fig.IV.3a), PE (30:0) ($p < 0.01$), and PE (32:0) ($p < 0.01$) (Fig. IV.3b) of cells exposed to the two different vancomycin concentrations in comparison to the control. lys-PG (30:0) and PE (30:0) content decreased significantly at lower vancomycin concentrations in comparison to the control, while PE (32:0) increased at higher vancomycin concentration in comparison to the control.

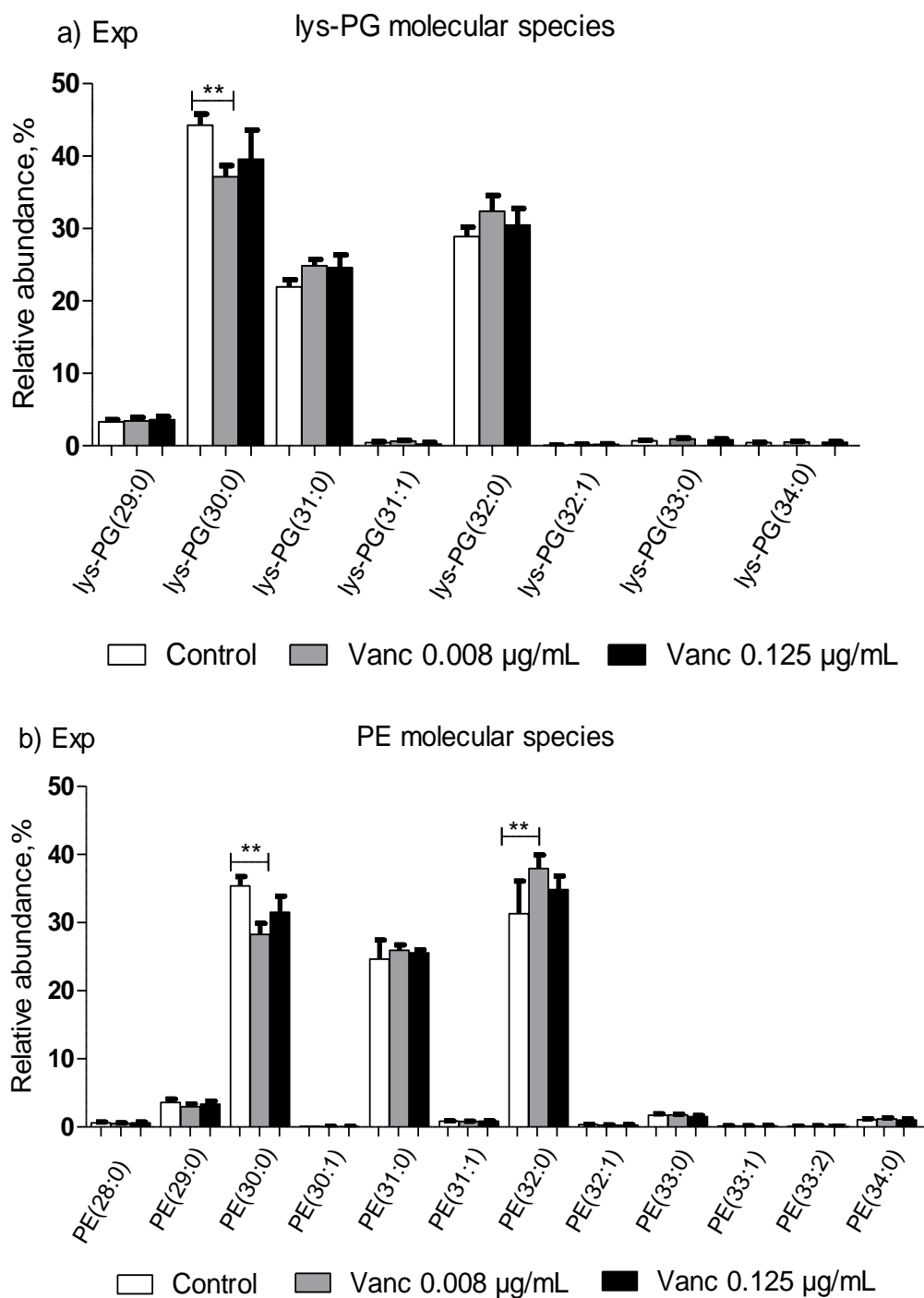


Figure IV.3. Variation of the polar lipid composition of *B. licheniformis* I89 membrane in the exponential phase, after exposure to vancomycin in concentrations of 0.008 and 0.125 µg/mL.

Variation was observed for the polar lipid classes lys-PG (a) and PE (b), Values are means \pm standard deviation, ** $p < 0.01$.

IV.4 Discussion

In the present study, we investigated the effect of the exposure to sub-inhibitory concentrations of vancomycin on the lipid content of *B. licheniformis* I89. The analyses were performed by HILIC-MS and GC-MS. Changes in the lipid composition have been observed in several bacteria that are resistant to antibiotics. Nonetheless, little is known about lipid composition in the presence of sub-inhibitory concentrations. Since *B. licheniformis* I89 is sensitive to vancomycin and considering that the mode of action of this antibiotic lies in the interaction with the cell wall, we expected to see changes in the lipid composition/content, since bacteria will be growing under some stress. To achieve this goal, cells were analyzed for their lipid polar and fatty acids contents after being grown in the presence of 0.008 and 0.125 $\mu\text{g/mL}$ of vancomycin. Lipid profile differences were also evaluated in the two different growth phases, lag and exponential.

Our preliminary study showed that polar lipids are modulated at small concentrations of antibiotic. Therefore, our objective was to understand the role of polar lipid in this context. Our results showed that in *B. licheniformis* I89 an adaption of the phospholipid metabolism after exposure to vancomycin has occurred, revealing significant changes in PG, lys-PG, PE and DGDG, mostly associated with a decrease of some PG (33:0 and 34:0) and lys-PG (32:0) species. In fact, major changes were observed specifically in the phospholipid profile, rather than in the glycolipid or primer of teichoic acid. In the cell membranes phospholipids regulate the spatial and temporal position and function of membrane proteins that play an essential role in a variety of cellular functions[12]. The detailed analysis of polar lipid classes here described revealed that different species of phospholipids respond distinctly to the exposure to vancomycin at 0.125 $\mu\text{g/mL}$. It appears that the adaptation of *B. licheniformis* I89 during exposure to vancomycin and lag phase of growth may be associated with a significant reduction of PG and lys-PG as well as DGDG. In addition, a decrease in lys-PG in the lag

phase was observed. Overall, our result shows alterations in the lipid composition in the presence of vancomycin, particularly with a reduction of PG.

In *B. subtilis* 168 and in the presence of sub-inhibitory concentrations of surfactin SF350, it appears that this lipopeptide induces a response both at the level of the polar heads and the fatty acids of membrane phospholipids [13].

Curiously, in *E. faecalis* and *E. faecium* a decrease in PG was also reported and it was associated with antibiotic resistance [13,14]. Furthermore, the importance of PG in the mechanism of bacterial resistance to antibiotics was already postulated by other researchers [15,16]. In fact, it is known that some antibiotics interact with PG and this is essential for the bactericidal action of such antibiotics, although this effect is still not well understood. It seems that a decrease in PG content could be a mechanism important for bacteria to develop resistance to a given antibiotic since the number of target molecules is reduced in the bacterial membrane. Besides, the amount of PE, and Lys-PG, in the membrane, can make it more susceptible/sensitive to antibiotic. Uttlová and co-workers analyzed alteration of the proportion of major membrane phospholipids in response to exposure to sub-inhibitory surfactin (SF 350 and SF 360) concentrations in *Bacillus subtilis* 168. They reported reduction in the content of PG and increase in PE, followed by higher levels of phosphatidic acid (PA) in the SF350 cultures [13].

Some studies also reported changes in the polar lipid profile as an adaptation mechanism of bacteria in the presence of an antibiotic. In some cases these changes are associated to antibiotic resistance [16–18]. The bacterial membrane is mainly composed of phospholipids that have a high net negative charge. Addition of Lys reduces the net negative charge of the cellular membrane and thus its affinity for cationic antimicrobial peptides (CAMPs), thereby increasing the resistance of the bacteria to these compounds [19]. Furthermore, in Gram-positive bacteria, the addition of D-Ala to the teichoic acid is an additional strategy used by microorganisms to resist CAMPs. [20]. In *E. faecalis* alteration in lipid homeostasis associated with daptomycin resistance was observed, with lower levels of PG and Lys-PG [16,18]. However, in *Staphylococcus aureus* and *Corynebacterium striatum* resistance to daptomycin, is associated with decrease of PG and Lys-PG and of cardiolipin and glycolipids [16].

It is accepted that FA influences the properties of the membrane, by regulating bacterial membrane homeostasis and interfering with cell adaptation to different environments, thus maintaining membranes properties, required for bacterial survival [21,22]. Gram-positive bacteria are characterized by a high content of branched-fatty acids, which were shown to be correlated to the activity of antimicrobial peptides. In fact, it was previously reported that in *S. aureus* changes in the fatty acid composition and higher content of anteiso-branched lipids can be associated with the resistance to antimicrobial agents. There is little information on how branched lipids and the location of the branching point affect the activity of active peptides in the membrane and bacterial cell wall [23]. In methicillin-resistant *S. aureus* (MRSA), the amount of anteiso-branched acyl chains is higher than that of susceptible strains. Furthermore, growth of *S. aureus* in medium containing sub-inhibitory concentrations of methicillin or antimicrobial peptides induces an increase in the anteiso-branched fatty acid fraction which are the major determinants of membrane fluidity [24]. Changes in the proportion of iso and anteiso FA have been reported in methicillin sensitive *S. epidermidis* and resistant *S. aureus* strains [25] and a significant increase in the anteiso/iso FA ratio accompanied by a decrease in saturated FA was observed in a daptomycin resistant strain when compared to the sensitive strain [26]. However, in the present study, the opposite was observed and *Bacillus* adaptation to vancomycin showed a significant decrease of i-17:0 in the lag phase that can lead to a decrease of membrane fluidity. However, this possibility still requires further study.

IV.4 Concluding remarks

In the present work, the analysis of membrane lipids showed that *B. licheniformis* I89 employs strategies to cope with this antimicrobial stress. We showed that in *B. licheniformis* I89 sub-inhibitory concentrations of antibiotics modulate the lipid metabolism, of bacteria cell wall phospholipids.

The most visible changes were in the PG, a negatively charged PL and Lys-PG, a positive PL. Both PG and lys-PG are important to maintain the net charge of the cell wall and are also

important targets for antibiotic binding. Thus, as vancomycin interacts with membrane phospholipids which may induce lipid profile alterations, this is an unexplored field that deserves additional studies.

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CHAPTER V

V. GENERAL CONCLUDING REMARKS

V. General Concluding Remarks

In this thesis, we advanced in the knowledge of the lipidome of the Gram-positive bacteria *B. licheniformis* I89 using modern lipidomics approaches, namely GC-MS and high-resolution HILIC-ESI-MS and MS/MS.

B. licheniformis I89 is a non-pathogenic Gram-positive bacterium, that has been highlighted for their biotechnological potential, foreseen by its capacity of producing several substances with commercial interest, such as lantibiotics among others. Here, we performed the first characterization of the lipidome of this bacterium through the identification of FA profile by GC-MS, and the characterization of the polar lipid profile by LC-MS. Lipids are the main components of the bacterial cell membranes and are important for cell survival. Lipid composition can shift depending on the growth conditions and are important to bacteria survival. Keeping this in mind, we also evaluated the variation of the lipidome, with regard to fatty acids and the polar lipids profiles, at different growth temperatures, in different growth phases and also the changes of the lipidome in response to the presence an antibiotic.

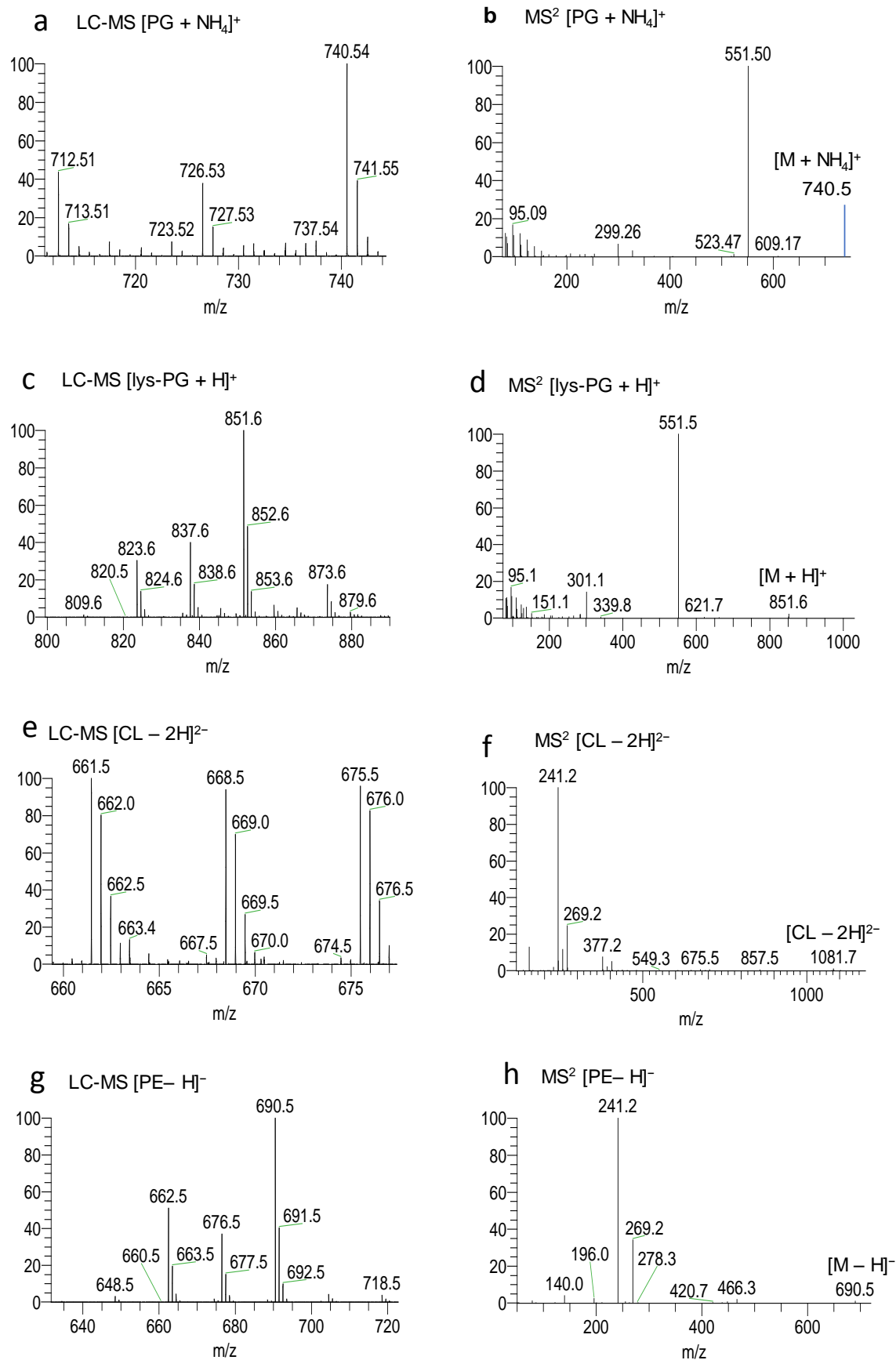
In chapter II, the fatty acid profile of *B. licheniformis* I89 was successfully characterized by GC-MS analysis. We have identified 9 of fatty acids where the most abundant were branched FAs iso- and anteiso C15 and C17, typical fatty acids found in *Bacillus* and in other Gram-positive bacteria. The results obtained also highlight an increase in the iso/anteiso FA ratio which is related to the increase of the growth temperature, at 37 °C and 50 °C, showing that even small variations of temperature affect the bacterial FA composition of *B. licheniformis* I89. The fatty acid profile was also influenced during the different growth phases. A higher relative content of ai-17:0 FA and lower i-15:0 were observed in the lag phase and a higher relative content of i-15: 0 e i-16:0 and lower relative content of ai-15: 0 e ai-17: 0 in the stationary phase.

In chapter III, new results obtained in the identification of the polar lipid profile are presented. This is the first complete study of the lipidome of a *Bacillus* species using high-resolution HILIC-ESI-MS approaches. This lipidomic approach based on high-resolution LC-MS/MS is nowadays the state of the art of the analytical methodology for the study of lipids, and to predict its application in different fields. The results gathered in this work allowed us to identify the complex full lipidome of *B. licheniformis* I89 that includes different types of lipids, such as phospholipids (PL), glycolipids (GL), and a

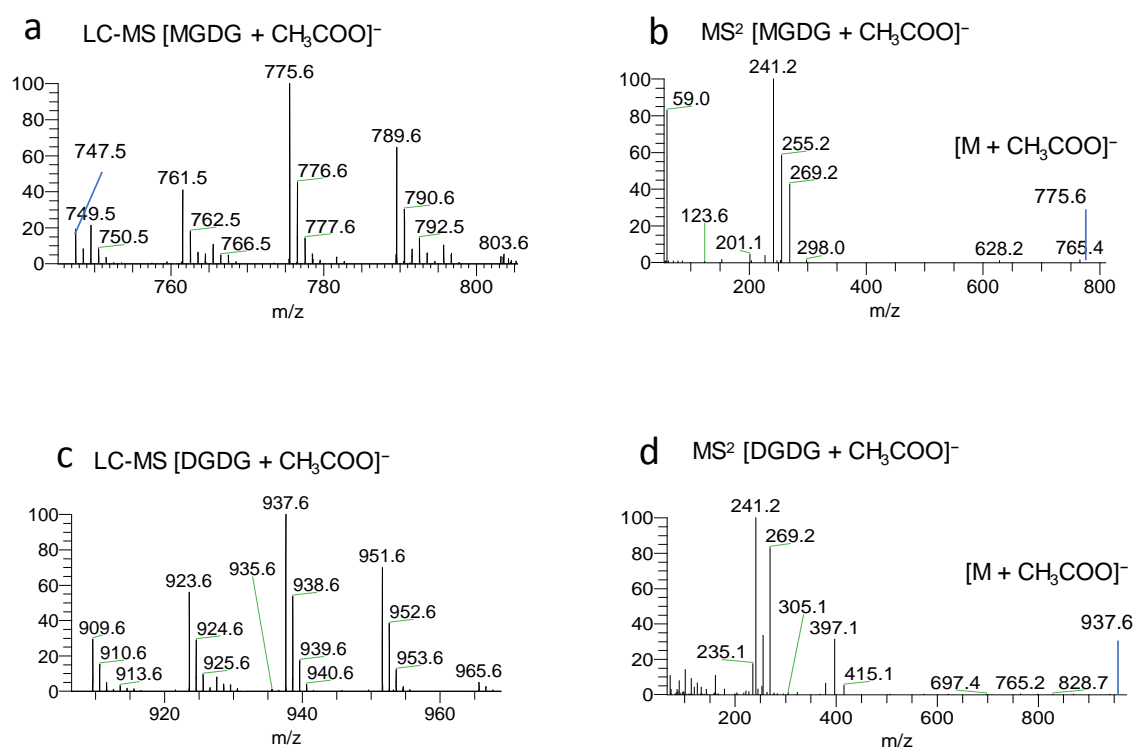
group of lipids specific, for bacteria, the lipoteichoic precursors, PGL. The lipidome study contributed greatly to the identification and structural characterization of the lipid composition of *B. licheniformis* I89. Detailed analysis at the molecular level allowed to identify the phospholipid PG, lys-PG, PE and CL, the glycolipids MDGD, DGDG, and the phosphoglyceroglycolipid LTAP, LTAP-Ala classes. In addition, the results revealed that in different growth phase the polar lipid profile showed some variation was found mainly in specific classes such as PG and GL, with an increase in the 30:0 lipids species and decrease in the 32:0 lipid species, between exponential and stationary phases in comparison to the lag phase.

In Chapter IV, since it is well known that membrane properties may vary when bacteria grow in the presence of antibiotics, although most of the work report the adaptation of other components of the membranes, such as peptides, or only FA. Herein we also show innovative results which show that there was a selective adaptation in the polar lipids profile when *B. licheniformis* I89 was cultured in the presence of sub-inhibitory concentrations of vancomycin. The results from by GC-MS and HILIC-ESI-MS analysis showed that under these conditions, the cells adapt their lipid metabolism of the membrane revealing a change of the amount of some lipid species of PG, PE, lys-PG and DGDG classes, during the lag phase and PE, lys-PG in the exponential phase. These polar lipids are important for maintaining the cell wall net charge and are also important for binding or preventing the action of antibiotics. Overall understanding the lipid adaptation and metabolism in bacteria is far from being completely understood but deserves to be considered for future research not only because some of the lipids, namely the branched FA, can have a biotechnological application but also the understanding of lipid remodelling in the presence of antibiotics can give new clues to develop new anti-microbial strategies.

APPENDIX A. SUPPLEMENTARY MATERIAL OF SECTION III



Supplementary fig. III.1 LC-MS spectra of the phospholipid classes (PLs) acquired in positive ion mode for PG (a), lys-PG (c), and in negative ion mode for PE(e) and CL(g), identified in *Bacillus licheniformis* I89 lipidome. LC-MS/MS spectra and possible fragmentation pattern of one of the possible isomers of the $[M + NH_4]^+$ ions of PG (17:0/15:0) at m/z 740.5(b), $[M + H]^+$ ion of Lys-PG (17:0/15:0) at m/z 851.6 (d), $[PE - H]^-$ ion of PE (17:1/15:0) at m/z 690.3(f) and $[CL - 2H]^{2-}$ ion of CL (15:0/17:0) m/z 661.5(h).



Supplementary fig. III.2 LC-MS spectra of the glycolipid classes (GLs) acquired in negative ion mode for MGDG (a), DGDG (c) identified in *Bacillus licheniformis* I89 lipidome. LC-MS/MS spectra and possible fragmentation pattern of one of the possible isomers of the $[M - CH_3COO]^-$ ions of MGDG (17:0/16:0) at m/z 775.6 (b) and DGDG (15:0/16:0) m/z 937.6(d).